BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/47, 31/30, 31/555 — A1

(11) International Publication Number: WO 96/39144

(43) International Publication Date: 12 December 1996 (12.12.96)

(21) International Application Number: PCT/US96/10122

(22) International Filing Date: 6 June 1996 (06.06.96)

(30) Priority Data:
08/468,645 6 June 1995 (06.06.95) US

(71) Applicant *(for all designated States except US)*: PROCYTE CORPORATION [US/US]; Suite 210, 12040-115th Avenue N.E., Kirkland, WA 98034-6900 (US).

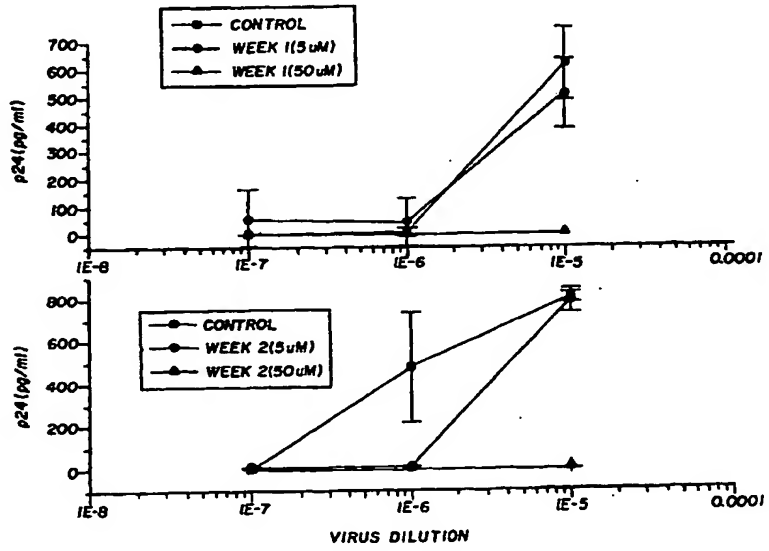
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PALLENBERG, Alexander, J. [US/US]; 20024-330th Avenue N.E., Duvall, WA 98019 (US). BRANCA, Andrew [US/US]; 1656 Goat Trail Loop Road, Mukilteo, WA 98275 (US). MARSCHNER, Thomas, M. [US/US]; Apartment H-203, 11229 N.E. 128th Street, Kirkland, WA 98034 (US). PATT, Leonard, M. [US/US]; 12016-40th Avenue N.E., Seattle, WA 98125 (US).

(74) Agents: HERMANNS, Karl, R. et al.; Seed and Berry L.L.P., 6300 Columbia Center, 701 5th Avenue, Seattle, WA 98104-7092 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: STABLE COPPER(I) COMPLEXES AS ACTIVE THERAPEUTIC SUBSTANCES



(57) Abstract

There is disclosed stable Copper(I) complexes and methods relating thereto. The stable Copper(I) complexes comprise a Copper(I) ion complexed by a multi-dentate ligand which favors the +1 oxidation state for copper. Uses of this invention include the use of the stable Copper(I) complexes as wound healing agents, anti-oxidative agents, anti-inflammatory agents, lipid modulating agents, signal transduction modulating agents, hair growth agents, and anti-viral agents. Uses of this invention also include inhibition of viral infection, as well as inhibiting transmission of sexually transmitted diseases. Exemplary stable Copper(I) complexes include neocuproine Copper(I) and bathocuproine disulfonic acid Copper(I).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

STABLE COPPER (i) COMPLEXES AS ACTIVE THERAPEUTIC SUBSTANCES

Technical Field

This invention is generally directed to a Copper(I) complex and methods relating to the use thereof and, more specifically, to Copper(I) complexed by a multi-dentate ligand such that the +1 oxidation state for copper is favored in the resulting complex.

Background of the Invention

Copper is found in both plants and animals, and a number of copper-containing proteins, including enzymes, have been isolated. Copper may exist in a variety of oxidation states, including the 0, +1, +2 and +3 oxidation states (i.e., copper(0), Copper(I), copper(II) and copper(III), respectively), with Copper(I) and copper(II) the most common. The relative stabilities of Copper(I) and copper(II) in aqueous solution depend on the nature of the anions or other ligands present in the solution. Moreover, only low equilibrium concentrations of Copper(I) in aqueous solutions (i.e., $< 10^{-2}$M) can exist. This instability is due, in part, to the tendency of Copper(I) to disproportionate to copper(II) and copper(0). Most Copper(I) compounds readily oxidize to copper(II) compounds, although further oxidation to copper(III) is difficult (*see, generally,* A.F. Cotton and G. Wilkinson, Advanced Inorganic Chemistry, 5th ed., John Wiley & Sons, New York, pp. 903-922, 1988).

Due to the relatively well-defined aqueous chemistry of copper(II), a large number of copper(II) salts and complexes are known. For example, a great deal of research has been directed to the biological activity of peptide/copper(II) complexes, and such copper(II)

complexes have been shown to possess utility for a variety of therapeutic and cosmetic purposes. In particular, the naturally occurring glycyl-histidyl-lysine:copper(II) complex ("GHK-Cu(II)") has been shown to be an effective agent in the enhancement of wound healing in warm-blooded animals, as well as generally serving as an anti-inflammatory agent (*see* U.S. Patent No. 4,760,051). Various derivatives of GHK-Cu(II) possess similar activity (*see* U.S. Patent Nos. 4,665,054 and 4,877,770). GHK-Cu(II) and other peptide-copper(II) complexes have also been shown to be effective for stimulating hair growth (U.S. Patent Nos. 5,177,061 and 5,120,831), for inducing biological coverings in wounds (U.S. Patent No. 4,810,693), for preventing ulcers (U.S. Patent Nos. 4,767,753, 5,023,237, 5,145,838), for cosmetic applications (U.S. Patent No. 5,135,913), and for healing bone (U.S. Patent No. 5,509,588). Moreover, anti-oxidative and anti-inflammatory activity of metal(II)-peptide complexes has been disclosed (U.S. Patent No. 5,118,665), as well as the use of copper(II)-containing compounds to accelerate wound healing (U.S. Patent No. 5,164,367).

Although great strides have been made in the study of copper(II) complexes, and particularly peptide/copper(II) complexes, there is still a need in the art for additional copper complexes which possess biological activity. The present invention fulfills this need, and provides further related advantages.

Summary of the Invention

This invention is generally directed to stable Copper(I) complexes and methods relating thereto. More specifically, the stable Copper(I) complexes of the present invention comprise Copper(I) complexed by a multi-dentate ligand such that the +1 oxidation state for copper is favored.

The stable Copper(I) complexes have utility for enhancing wound healing in warm-blooded animals, for enhancing or restoring the resistance of warm-blooded animals to oxidative or inflammatory damage associated with reactive oxygen species and/or lipid mediators, for stimulating the growth of hair in warm-blooded animals, for modulating lipid metabolism, for modulating signal transduction in cells by inhibiting protein kinases, and for inhibiting viral activity and infection, including (but not limited to) HIV replication in an HIV-infected animal. Methods of the present invention comprise administering an effective amount of a stable Copper(I) complex to the animal.

Other aspects of this invention will become evident upon reference to the attached figures and following detailed description. All references identified in the detailed description, including the examples, are hereby incorporated by reference in their entirety

Description of the Figures

Figure 1 illustrates the activity of a representative Copper(I) complex of this invention (i.e., bathocuproine disulfonic acid ("BCDS") Copper(I)) to accelerate wound healing.

Figure 2 illustrates the ability of a representative Copper(I) complex of the present invention, BCDS Copper(I), to inhibit viral (i.e., HIV) replication.

Figure 3 illustrates synthesis pathways for prostaglandins and leukotrienes, as well as certain key enzymes associated therewith.

Figure 4 illustrates a synthesis pathway for cholesterol formation, including the intermediates acetyl CoA and HMG-CoA and the enzymes acetyl CoA synthetase and HMG-CoA reductase.

Figure 5 illustrates the action of Protein Kinase C (PKC) and protein tyrosine kinase in signal transduction

(PI = phosphatidyl inositol, IP^3 = inositol triphosphate, PG = phosphatyl glycerol, P-Protein = phosphorylated protein, CDR PK = calmoduln-regulated protein kinase, PKA = Protein Kinase A, Protein Kinase = Protein Tyrosine Kinase (cytoplasmic), and EGF-R Protein Kinase = Epidermal growth factor receptor protein tyrosine kinase).

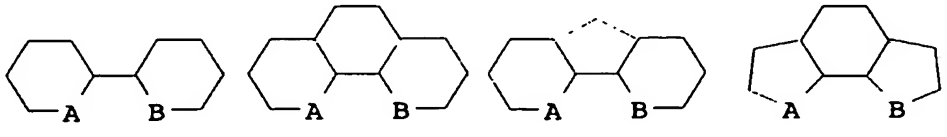
Detailed Description

This invention is generally directed to Copper(I) complexes and methods relating to the use thereof, and more specifically, to Copper(I) complexed by a multi-dentate ligand to form a stable Copper(I) complex. As used herein, a "stable Copper(I) complex" is Copper(I) chelated by at least one multi-dentate ligand such that the resulting complex favors the +1 oxidation state of copper. The most common states of Copper(I) are associated with four coordination sites, and are generally of a tetrahedral configuration. In general, chelating agents are coordination compounds in which a single ligand occupies more than one coordination position of a metal ion. If the ligand occupies two coordination positions, it is considered a bi-dentate ligand; if more than two coordination positions are occupied by the ligand, it is considered a poly-dentate ligand (such as a tri-dentate ligand or a tetra-dentate ligand). As used herein, a "multi-dentate ligand" is a bi-, tri- or tetra-dentate ligand which occupies two, three or four coordination sites, respectively, of copper (I).

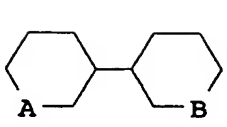
The stable Copper(I) complexes of this invention include all complexes of Copper(I) chelated by at least one multi-dentate ligand which structurally favors the +1 oxidation state of copper. Copper(I) complexes may be formed by reacting a multi-dentate ligand with a source of Copper(I) (such as CuCl, Cu_2O or CuCN) in aqueous solution. The resulting Copper(I) complex may then be observed by suitable analytical techniques, such as ESR, NMR and/or

UV-VIS, to determine the oxidation state of the copper in the complex (*see* Munakata et al., Copper Coordination Chemistry: Biochemical and Inorganic Perspectives, Karlin and Zubieta editors, Adenine Press, Guilderland, N.Y., pp. 473-495, 1983). For example, Copper(I) complexes can be identified by their characteristic absence of an ESR signal, while copper(II) complexes will generally possess an ESR signal. Furthermore, copper(II) complexes exhibit broadening of proton NMR signals, and Copper(I) complexes exhibit relatively sharp proton NMR signals. Following identification of the Copper(I) complex, its stability can be evaluated by determining its susceptibility to oxidation by, for example, exposing the Copper(I) complex to air. As used herein, a "stable" Copper(I) complex has a half-life of at least 5 minutes, preferably of at least one hour, and more preferably of 24 hours or more (i.e., half of the Copper(I) complex remains in the +1 oxidation state) upon exposure to air, at room temperature (23°C) and atmospheric pressure. In other words, stable Copper(I) complexes of this invention resist oxidation, while non-stable Copper(I) complexes are readily oxidized to yield copper(II) complexes upon exposure to air.

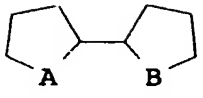
As mentioned above, any multi-dentate ligand which chelates Copper(I) to yield a stable Copper(I) complex is suitable in the practice of this invention. However, in a preferred embodiment, the multi-dentate ligands of this invention are selected from the following general structures I through VII:



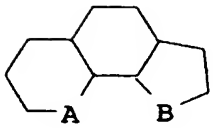
I II III IV



V



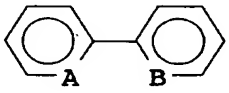
VI



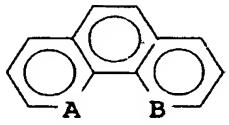
VII

wherein A and B represent heteroatoms which may occupy coordination sites of Copper(I), and are preferably selected from nitrogen, oxygen, sulfur and phosphorous.

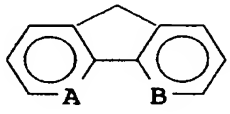
The rings of structures I through VII may be aromatic, non-aromatic or a mixture of both aromatic and non-aromatic rings. For example, the following structures are representative of such combinations:



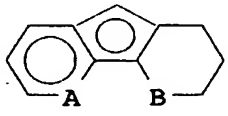
Ia



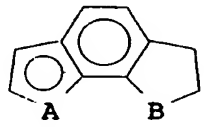
IIa



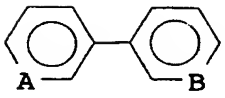
IIIa



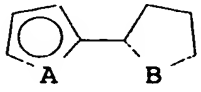
IIIa'



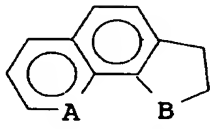
IVa



Va



VIa

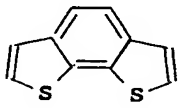
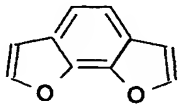
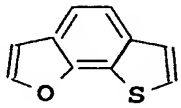
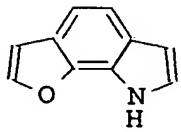
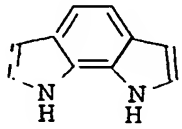
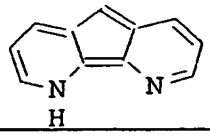
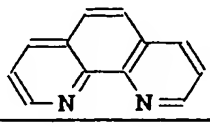


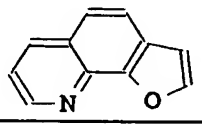
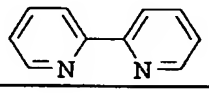
VIIa

Representative examples of multi-dentate ligands of this invention having structures I through VII are set forth in Table 1. Specifically, Table 1 identifies the structure of the representative multi-dentate ligand, lists the corresponding chemical name, identifies the Chemical Abstracts Registration Number ("CA Reg. No."), and provides a corresponding reference (if available)

describing the synthesis and/or chemistry of the identified multi-dentate ligand.

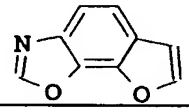
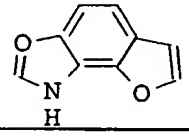
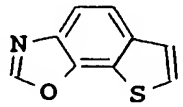
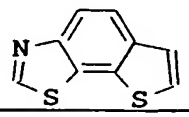
Table 1

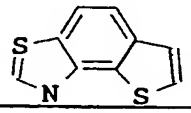
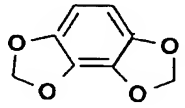
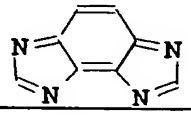
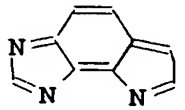
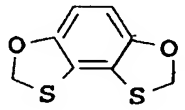
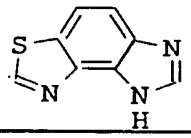
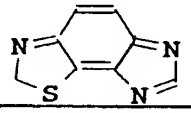
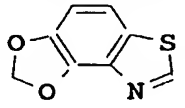
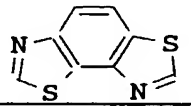
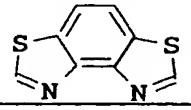
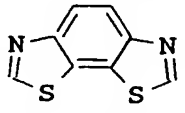
Structure	Name	CA Reg. No.	Reference
	benzo (2,1-b:3,4-b) dithiophene	211-53-0	Sturaro et al., Heterocycl. Chem. 27:1867, 1990
	benzo (2,1-b:3,4-b) difuran	211-47-2	Rene et al., Eur. J. Med. Chem.-Chim. Ther. 13:435, 1978
	thieno (3,2-g) benzofuran	438-31-9	Cagniant and Kirsch, Hebd. Seances Acad. Sci. C. 282:465, 1976
	2H-furo(3,2-g) indole	103671-62-1	Lawrence Jr., Eur. Pat. Appl. EP 173,520, 1986
	2H-benzo (2,1-b:3,4-b') dipyrrole	112149-08-3	Berlin et al., J. Chem. Soc. Chem. Commun. (15):1176, 1987
	1H-cyclopenta (2,1-b:3,4-b') bipyridine	42262-29-3	
	1,10-phenanthroline	66-71-7	

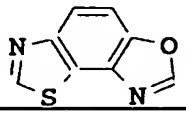
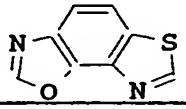
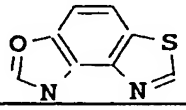
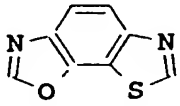
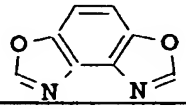
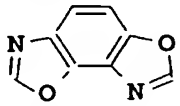
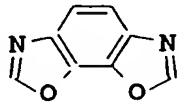
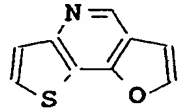
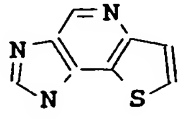
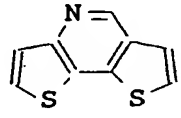
	furo (3,2-h) quinoline	234-28-6	
	2,2'-bipyridyl	366-18-7	

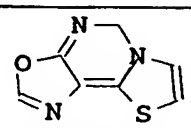
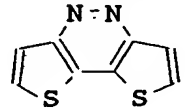
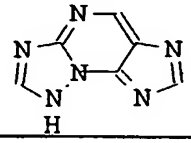
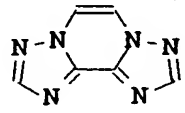
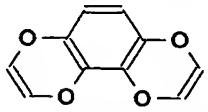
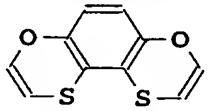
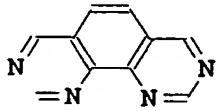
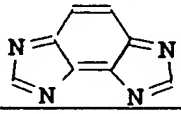
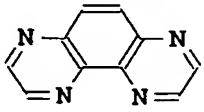
In structures I through VII above, further ring substitutions with heteroatoms are permitted. Preferably, such heteroatoms are selected from nitrogen, oxygen, sulfur, and phosphorus. For example, the compounds listed in Table 2 illustrate further representative multi-dentate ligands of the present invention having additional ring substitutions. As with Table 1, Table 2 identifies the structure of the representative multi-dentate ligands, lists the corresponding chemical name, identifies the CA Reg. No., and provides a corresponding reference (if available) describing the synthesis and/or chemistry of the identified multi-dentate ligand.

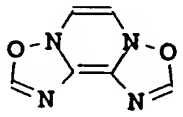
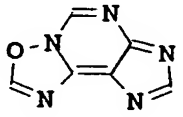
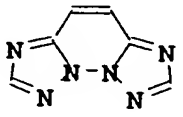
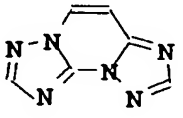
Table 2

Structure	Name	CA Reg. No.	Reference
	furano (3,2-g) benzoxazole	25885-39-6	
	furano (2,3-e) benzoxazole	66037-80-1	Turin et al., Fr. Demande 2,338,041, 1977
	thieno (3,2-g) benzoxazole	58188-85-5	Iddon et al., J. Chem. Soc., Perkin Trans. I 17:1686, 1975
	thieno (3,2-g) benzothiazole	72121-58-5	

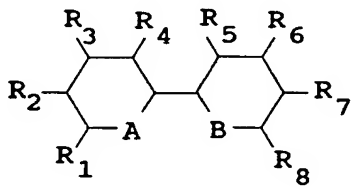
	thieno (2,3-e) benzothiazole	211-36-9	
	benzo (1,2-d:3,4-d') bis (1,3) dioxide	211-50-7	Dallacker and Weiner, Justus Liebigs Ann. Chem. 725:99, 1969
	benzo (1,2-d:3,4-d') diimidazole	211-10-9	
	pyrrolo(2,3-e) benzimidazole	53068-46-5	Chetverikov et al., U.S.S.R. 425,906, 1974
	benzo (2,1-d:3,4-d') bis (1,3) oxathiole	211-54-1	
	2H-imidazo (4,5-e) benzothiazole	42341-40-2	
	2H-imidazo (4,5-g) benzothiazole	211-23-4	
	1,3-dioxolo (4,5-e) benzothiazole	77482-58-7	Foerster et al., Ger. Offen. 2,903,966, 1980
	benzo (1,2-d:3,4-d') bisthiazole	211-37-0	
	benzo (2,1-d:3,4-d') bisthiazole	23147-19-5	
	benzo (1,2-d:4,3-d') bisthiazole	10558-80-2	Grandolini et al., Ann. Chim. 58:91, 1968

	thiazolo(5,4-e) benzoxazole	211-35-8	
	thiazolo (5,4-g) benzoxazole	51273-21-3	
	thiazolo (4,5-e) benzoxazole	315-47-9	
	thiazolo (4,5-f) benzoxazole	67239-73-0	Fridman et al., Ikr. Khim. Zh. 44:399,1978
	benzo (2,1-d:3,4-d') bisoxazole	211-19-8	
	benzo (1,2-d:3,4-d') bisoxazole	211-20-1	
	benzo (1,2-d:4,3-d') bisoxazole	54935-19-2	Barker et al., J. Chem. Res. Synop. (9):328, 1986
	furo (2,3-d) thieno (3,2-b) pyridine	110665-19-5	
	1H-imidazo (4,5-d) thieno (3,2-b)-pyridine	111163-54-3	Takada et al., Eur. Pat. Appl. EP 223,420, 1987
	dithieno (3,2-b:2',3'-d) pyridine	40826-38-8	Yang et al., Synthesis 2:130, 1989; Heeres et al., Syn. Commun. 2:365, 1972

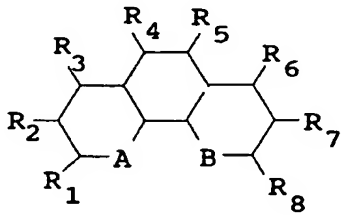
	5H-oxazolo (4,5-e) thiazolo (3,2-c) pyrimidine	211-46-1	
	dithieno (3,2-c:2',3'-e) pyridazine	51974-92-6	Nonciaux et al., Bull. Soc. Chim. Fr. 12 Pt 2, 3318, 1973
	1H-(1,2,4) triazolo (5,1-b) purine	387-96-2	
	bis (1,2,4) triazolo (1,5-d:5',1'-c) pyrazine	55366-22-8	Vercek et al., Tetrahedron Lett. (51/52):4539, 1974
	benzo (2,1-b:3,4-b') dipyran	231-29-8	Monatsch 80:743, 1949
	benzo (1,2-b:4,3-b') bis (1,4)-oxathiin	231-34-5	
	benzo (1,2-e:3,4-e') dipyrazine		
	benzo (1,2-d:3,4-d') diimidazole	211-10-9	
	pyrazino (2,3-f) quinoxaline	231-23-2	Shim et al., Synthesis 2:116, 1980; Nasielski-Hinkins et al., J. Chem. Soc. Perkin Trans. 1:1229, 1975

	bis (1,2,4) oxadiazolo (2,3-d:3',2'-c) pyrazine	74382-83-5	
	(1,2,4)-oxadiazolo (3,2-i) purine	56248-95-4	Miura et al., Chem. Pharm. Bull. 23:464, 1975
	bis (1,2,4) triazolo (1,5-b:5',1'-f) pyridazine	51519-32-5	Polanc et al., J. Org. Chem. 39:2143, 1974
	bis (1,2,4) triazolo (1,5-d:1',5'-c) pyrimidine	76044-62-7	Brown and Shinozuka, Aust. J. Chem. 33:1147, 1980

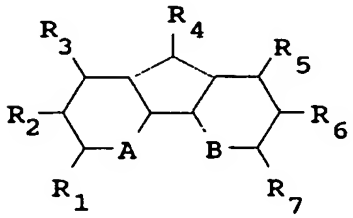
General structures I through VII identified above may possess further chemical moieties covalently attached to the structural backbone, as illustrated below:



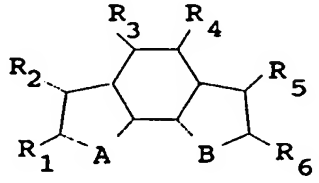
Ib



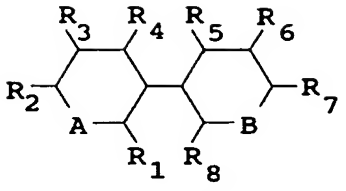
IIb



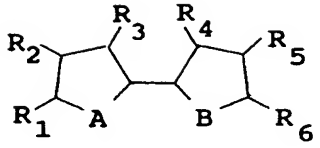
IIIb



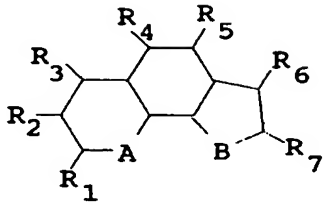
IVb



Vb



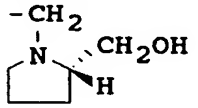
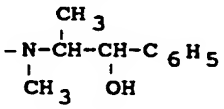
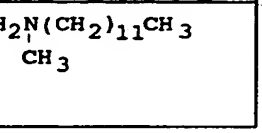
VIb



VIIb

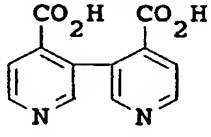
wherein R_1 through R_8 are the same or different, and are selected from the following chemical moieties: -H, -OH, -X, -OX, -COOH, -COOX, -CHO, -CXO, -F, -Cl, -Br, -I, -CN, $-NH_2$, -NHX, $-NX_2$, $-PX_2$, $-SO_3H$, $-SO_3Na$, $-SO_3K$, $-SO_3X$, $-PO_3H$, $-OPO_3H$, $-PO_3X$, $-OPO_3X$ and $-NO_2$. As used herein, "X" represents and an alkyl moiety or an aryl moiety. An "alkyl moiety" is a straight chain or branched, cyclic or noncyclic, saturated or unsaturated, substituted or unsubstituted carbon chain containing from 1-20 carbon atoms; and an "aryl moiety" is a straight chain or branched, cyclic or noncyclic, saturated or unsaturated, substituted or unsubstituted carbon chain containing at least one substituted or unsubstituted aromatic moiety and containing from 6-20 carbon atoms. Such chemical moieties may also be covalently attached to the ring fusion atoms. Representative examples of the chemical moieties of this invention include, but are not limited to, the moieties identified in Table 3 below.

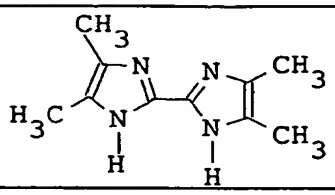
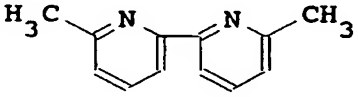
Table 3

-H	$-CH_3$	$-CH_2Br$
$-CH_2OH$	$-CH_2Cl$	$-CBr_3$
$-CH_2C_6H_5$	$-C_6H_5$	$-(CH_2)_{1-12}CH_3$
-Cl	-CHO	-COOH
-COOMe	-CH=NOH	$-CH_2NH_2$
$-CH_2C{\equiv}CH$	$-CH{=}CH_2$	$-P(C_6H_5)_2$
$-CH_2CH(CO_2H)_2$	$-CON(CH_2COOH)_2$	$-CH_2N(CH_2COOH)_2$
		
$-Ph-SO_3Na$		

Representative examples of the multi-dentate ligands possessing further chemical moieties covalently attached to the structural backbone of structures I through VII are presented in Table 4. In particular, Table 4 identifies the structure of the representative multi-dentate ligands, lists the corresponding chemical name, identifies the CA Reg. No., and provides a corresponding reference (if available) describing the synthesis and/or chemistry of the multi-dentate ligand.

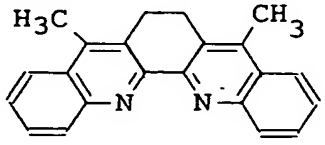
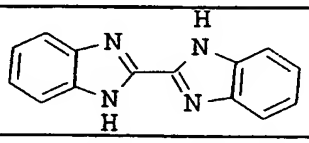
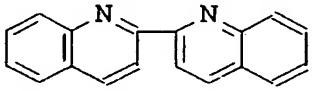
Table 4

Structure	Name	CA Reg. No.	Reference
	2,2'-bipyridine-4,4'-dicarboxylic acid	6813-38-3	

	2,2'-bis (4,5-dimethyl imidazole)	69286-06-2	J. Organomet. Chem. 307:39, 1986
	2,3-bis (2-pyridyl) pyrazine	25005-96-3	(Aldrich: 28,164-16)
	5,5'-dimethyl-2,2'-bithiophene	16303-58-5	
	6,6'-dimethyl-2,2'-dipyridine	4411-80-7	Kauffmann et al., Chem. Ber. 109:3864, 1976

The chemical moieties covalently attached to the structural backbone may be joined to yield an aromatic or nonaromatic cyclic chemical moiety. Representative examples of such cyclic chemical moieties are set forth in Table 5, which identifies the structure of the representative multi-dentate ligands, lists the corresponding chemical name, identifies the CA Reg. No., and provides a corresponding reference (if available) describing the synthesis and/or chemistry of the multi-dentate ligand.

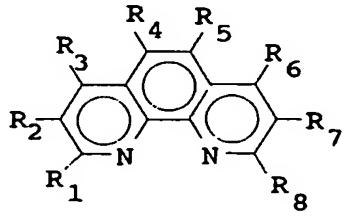
Table 5

Structure	Name	CA Reg. No.	Reference
	6,7-dihydro-5,8-dimethyl dibenzo (b)(1,10) phenanthroline	5298-71-5	
	bibenzimidazole	123067-51-6	
	2,2'-bisquinoline	119-91-5	(Aldrich: B3,540-7)

The synthesis of representative examples of the multi-dentate ligands of this invention are disclosed in Table 6 and Table 7 below. Specifically, in these tables the structure of the multi-dentate ligands are identified along with their CA Reg. No. and one or more references disclosing their synthesis and/or chemistry.

Table 6

Synthesis of Representative Copper(I) Complexes Having the Structure:



(R_2 through R_7 = hydrogen, unless indicated)

R1	R8	CA Reg. No.	Reference
$-CH_3$	$-CH_3$	484-11-7	O'Reilly et al., Aust. J. Chem. 13:145, 1960
$-CH_2Br$	$-CH_2Br$	78831-37-5	Weijen et al., J. Org. Chem. 57:7258, 1992; Jukkala et al., Helv. Chim. Acta. 75:1621, 1992; Chandler et al., J. Heterocycl. Chem. 18:599, 1981
$-CH_2Br$	$-CH_2OH$	142470-16-4	Weijen et al., J. Org. Chem. 57:7258, 1992
$-CBr_3$	$-CBr_3$		Chandler et al., J. Heterocycl. Chem. 18:599, 1981
$-CH_2Cl$	$-CH_2Cl$		Newkome et al., J. Org. Chem. 50:3807, 1985; Newcome et al., J. Org. Chem. 48:5112, 1983
$-CCl_3$	$-CCl_3$		Chandler et al., J. Heterocycl. Chem. 18:599, 1981; Newcome et al., J. Org. Chem. 48:5112, 1983
$-CN$	$-CN$	57709-63-4	Chandler et al., J. Heterocycl. Chem. 18:599, 1981; Sjoegren et al., Organometallics 11:3954, 1992
$-CH_2C_6H_5$	$-CH_2C_6H_5$	223-20-1	Sjoegren et al., Organometallics 11:3954, 1992
$-(CH_2)_{11}CH_3$	$-(CH_2)_{11}CH_3$		Menger et al., J. Am. Chem. Soc. 113:4017, 1991
$-(CH_2)_3CH_3$ (R_3=R_6=H, Ph	$-(CH_2)_3CH_3$	85575-93-5P	Sugihara et al., JP 02096578 A2, Jpn. Kokai Tokkyo Koho 113(15):132159v
$-(CH_2)_3CH_3$ (R_4=R_5=$-CH_3$)	$-(CH_2)_3CH_3$		Delton et al., EP 339973 A1, Eur. Pat. Appl. 112(21):19835p, 1989

-Cl	-Cl	29176-55-4	Sjoegren et al., Organometallics 11:3954, 1992; Delton et al., EP 339973 A1, Eur. Pat. Appl. 112(21):19835p, 1989
$-CH_2OH$	$-CH_2OH$	78831-36-4	Chandler et al., J. Heterocycl. Chem. 18:599, 1981; Delton et al., EP 339973 A1, Eur. Pat. Appl. 112(21):19835p, 1989; Newcome et al., J. Org. Chem. 48:5112, 1983
-CHO	-CHO	57709-62-3	Ziessel, Tetrahedron Lett. 30:463, 1989; Toner, EP 288256 A2, Eur. Pat. Appl. 111(15):130322c; Bell et al., J. Inclusion Phenom. 5:149, 1987
-COOH	-COOH		Chandler et al., J. Heterocycl. Chem. 18:599, 1981
-COOMe	-COOMe		Chandler et al., J. Heterocycl. Chem. 18:599, 1981; Newcome et al., J. Org. Chem. 48:5112, 1983
-CH=NOH	-CH=NOH		Chandler et al., J. Heterocycl. Chem. 18:599, 1981
$-CH_2NH_2$	$-CH_2NH_2$		Chandler et al., J. Heterocycl. Chem. 18:599, 1981
-CHO	-H	33795-37-8	Toner, EP 288256 A2, Eur. Pat. Appl. 111(15):130322c
-COOH	-H	1891-17-4	Toner, EP 288256 A2, Eur. Pat. Appl. 111(15):130322c
$-CH_2C{\equiv}CH$	$-CH_2C{\equiv}CH$		Sjoegren et al., Organometallics 11:3954, 1992
$-C_6H_5$	$-C_6H_5$		Dietrich-Buchecker et al., Tetrahedron Lett. 23:5291, 1982
-Cl	$-CH_3$		Newcome et al., J. Org. Chem. 54:1766, 1989
$-CH=CH_2$	$-CH=CH_2$		Newkome et al., J. Org. Chem. 50:3807, 1985
$-P(C_6H_5)_3$	$-P(C_6H_5)_3$		Ziessel, Tetrahedron Lett. 30:463, 1989

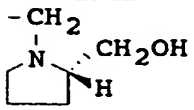
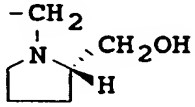
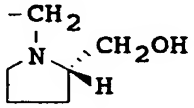
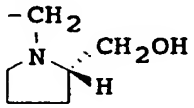
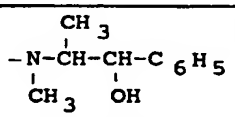
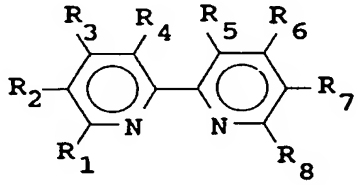
$CH_2CH(CO_2H)_2$	$-CH_2CH(CO_2H)_2$		Newcome et al., Inorg. Chem. 24:811, 1985
$-CH_2N(CH_2)_{11}CH_3$ CH_3			Weijen et al., J. Org. Chem. 57:7258, 1992
			Weijen et al., J. Org. Chem. 57:7258, 1992
$-CH_2OH$			Weijen et al., J. Org. Chem. 57:7258, 1992
$-CH_2N(CH_2)_{11}CH_3$ CH_3			Weijen et al., J. Org. Chem. 57:7258, 1992
$-CH_2N(CH_2COOH)_2$	$-CH_2N(CH_2COOH)_2$		Mukkala et al., Helv. Chim. Acta 75:1621, 1992; Toner, EP 288256 A2, Eur. Pat. Appl. 111(15):130322c
$-CON(CH_2COOH)_2$	$-CON(CH_2COOH)_2$		Toner, EP 288256 A2, Eur. Pat. Appl. 111(15):130322c
$-CH_3$ (R_3=R_6= $-Ph-SO_3Na$	$-CH_3$	52698-84-7	Blair et al, Talanta 7:163, 1961

Table 7

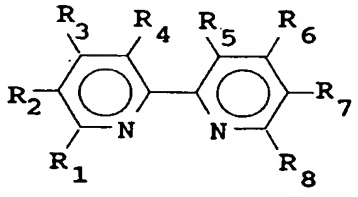
Synthesis of Representative Copper(I) Complexes Having the Structure:



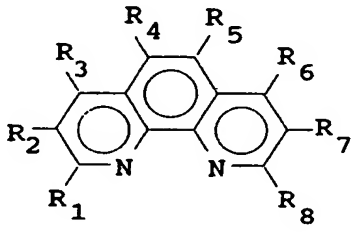
(R_2 through R_7 = hydrogen, unless indicated)

R1	R8	CA Reg. No.	Reference
-CN	-CN	4411-83-0	Sjoegren et al., Organometallics 11:3954, 1992
$-CH_2Cl$	$-CH_2Cl$	74065-64-8	Bell et al., J. Inclusion Phenom. 5:149, 1987
-CHO	-CHO		Newkome et al., J. Org. Chem. 50:3807, 1985
$-CH=CH_2$	$-CH=CH_2$		Newkome et al., J. Org. Chem. 50:3807, 1985
(R_1 and R_2= benzo moiety)	(R_7 and R_8 = benzo moiety)	119-91-5	(Aldrich: B3,540-7)

In one embodiment of this invention, the multi-dentate ligands are selected from the following structures:



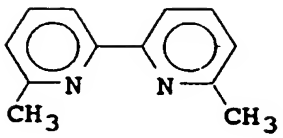
Ic



IIc

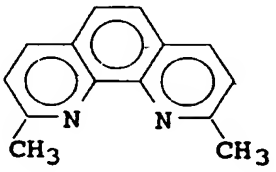
wherein R_1 through R_8 are the same or different, and are selected from hydrogen, an alkyl moiety and an aryl moiety.

In a preferred embodiment, the multi-dentate ligand is 6,6'-dimethyl-2,2'-dipyridine having structure Id:

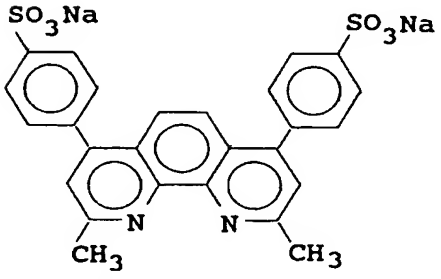


Id

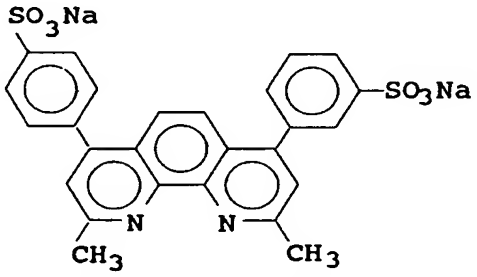
In a further preferred embodiment, the multi-dentate ligand is neocuproine (2,9-dimethyl-1,10-phenanthroline) having structure IId, or is bathocuproine disulfonic acid ("BCDS") having one of the isomeric structures IIe, IIe', IIe'' or IIe''':



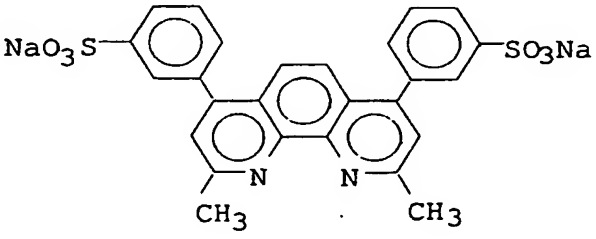
IId



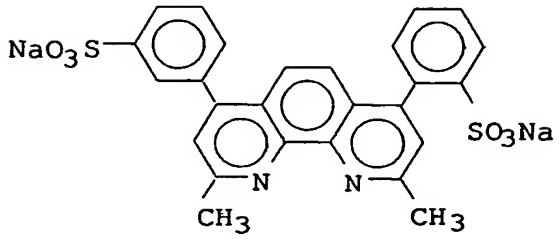
IIe (para,para)



IIe'(meta, para)



IIe'' (meta, meta)



IIe''' (ortho, meta)

Unless otherwise indicated, BCDS refers to a physical mixture of the above isomers (i.e., IIe, IIe', IIe'' and IIe'''). Typically, the ratio of the various isomers (i.e., IIe:IIe':IIe'') vary depending upon the commercial source of BCDS as follows: Aldrich Chemical Co., Inc. (Milwaukee, Wisconsin) 9.1:38.6:41.2; Spectrum Chemical Manufacturing Corp. (Gardena, California) 8.5:39.7:45.2; GFS Chemicals (Columbus, Ohio) 8.4:38.5:45.3; Janssen Pharmaceutica (subsidiary of Johnson & Johnson) (Beerse, Belgium) 4.6-8.7:36.4-39.4:44.4-55.9; with the IIe''' isomer present in the commercial source in only trace amounts (*i.e.*, typically about 1%).

As discussed above, stable Copper(I) complexes of this invention may be made by contacting a multi-dentate ligand with a Copper(I) source. The multi-dentate ligands may be obtained from commercial sources, or may be synthesized by known organic synthesis techniques from commercially available reagents. Preferably, water soluble multi-dentate ligands are complexed with the Copper(I) in aqueous solution, employing CuCl, Cu_2O or CuCN as the Copper(I) source. The resulting Copper(I) complex may then be recovered by evaporation of solvent to yield the Copper(I) complex. Alternatively, if the multi-dentate ligand is not readily soluble in water, Copper(I) complexes may be formed by the above procedure employing a suitable non-aqueous (e.g., organic) solvent.

In the practice of this invention, the ratio of the multi-dentate ligand to Copper(I) may be any ratio which results in a stable Copper(I) complex. Preferably, the ligand to copper ratio is at least 1:1. In a more preferred embodiment, the ligand to copper ratio ranges from 1:1 to 3:1 (including 2:1). Such Copper(I) complexes may be made by the procedures identified in the preceding paragraph by reacting the appropriate molar ratios of the multi-dentate ligand and the Copper(I) ion source.

Although not intending to be limited by the following theory, it is believed that Copper(I) has enhanced biological activity over copper(II) in certain biological events. For example, it is believed that Copper(I) may be an important intermediate for copper metabolism, including copper uptake and/or transfer, as well as cellular delivery. Thus, the reduction of copper(II) to Copper(I) is bypassed by direct delivery of Copper(I). Furthermore, the stable Copper(I) complexes of this invention are suitable for systemic delivery to warm blooded animals, and may provide a sustained release of copper to the animal.

The stable Copper(I) complexes of this invention possess utility as therapeutic substances, including utility as anti-oxidative and anti-inflammatory agents generally and, more specifically, as wound healing agents. The Copper(I) complexes of this invention also possess activity as hair growth agents, lipid modulation agents, signal transduction modulating agents, and anti-viral agents. For purpose of clarity, the various biological activities of the stable Copper(I) complexes of this invention are addressed individually below.

Highly reactive oxygen species such as the superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and lipid peroxides (LOOH) are involved in a number of human diseases. For example, such oxygen species have been implicated in autoimmune diseases, arthritis, tissue

damage caused by environmental pollutants, cigarette smoke and drugs, tissue injury during, for example, surgery and transplantation, as well as a variety of other conditions (see, e.g., Halliwell, B., Fed. Amer. Soc. Exp. Biol. 1:358-364, 1987). Reactive oxygen species are also generated during the response to injury by phagocytic cells. One of the early events in the wound healing response is the cleansing and sterilization of the wound by neutrophils and macrophages. A mechanism for this sterilization is the generation of the superoxide anion and hydrogen peroxide, and generally results in an inflammatory response. Moreover, superoxide anion and hydrogen peroxide will, in the presence of iron or other redox active transition metal complexes, generate the hydroxyl radical. The hydroxyl radical is a potent oxidant which initiates the free radical oxidation of fatty acids, as well as the oxidative degradation of other biomolecules. For example, an important area in which reactive oxygen species cause tissue damage is in post-injury damage to the brain and spinal chord, and in reperfusion injury to ischemic tissue following surgery and transplantation (such as heart surgery and/or transplantation). A sudden inrush of oxygenated blood and activated phagocytic cells leads to superoxide anion and hydrogen peroxide formation. These species do direct damage to tissue, and also react with iron (as discussed above) to generated the very reactive hydroxyl radical.

The stable Copper(I) complexes of this invention generally serve as anti-oxidative agents which prevent or limit the oxidative damage caused by reactive oxygen species, and further serve as anti-inflammatory agents by reducing the inflammatory response associated with such reactive oxygen species. More specifically, the Copper(I) complexes of the present invention are useful in the enhancement and/or restoration of the defense of warm-blooded animals to oxidative or inflammatory damage caused

by the highly reactive oxygen species, and may be used in pharmaceutical preparations to inhibit oxidative and inflammatory processes which lead to tissue damage. Moreover, the stable Copper(I) complexes of this invention accelerate the wound healing process by "detoxifying" tissue damage by the highly reactive oxygen species.

In addition to highly reactive oxygen species, macrophages and neutrophils induce or continue an inflammatory response through the generation of certain lipid mediators of inflammation (e.g., leukotrienes and prostaglandins). The involvement of such mediators in inflammatory bowel disease (IBD) and related chronic inflammatory conditions, such as arthritis, is evidenced by a strong correlation between disease progression and the levels and presence of leukotrienes and prostaglandins in the circulation and effected tissue. Prostaglandins enhance vasodilation and edema formation, while leukotrienes are potent chemoattractive agents for leukocytes, especially neutrophils, and stimulate degranulation and the release of damaging lysosomal enzymes and superoxide production.

The distribution of the two major pathways leading either to prostaglandins or to leukotrienes varies according to cell type. While most cells possess the cyclooxygenase pathway, the 5-lipoxygenase pathway leading to the leukotrienes is less widely distributed and is prominent in inflammatory cells, such as neutrophils, macrophages, monocytes and mast cells. The general scheme for lipid mediator synthesis is illustrated in Figure 3.

The stable Copper(I) complexes of this invention inhibit the formation of prostaglandins and/or leukotrienes by inhibiting the enzymes involved in their formation. Referring to Figure 3, the stable Copper(I) complexes are effective inhibitors of both cyclooxygenase-1 and cyclooxygenase-2, thereby inhibiting the formation of prostaglandins. Similarly, the stable Copper(I)

complexes are effective inhibitors of 5-lipoxygenase and leukotriene C_4 (LCT_4) synthetase, thereby inhibiting the formation of leukotrienes.

In addition, proteolysis of various cellular targets by elastase (a neutrophil-released serine protease) at the site of inflammation has been implicated in a number of pathologic conditions, including emphysema, rheumatoid arthritis, and psoriasis. Thus, inhibitors of elastase may be used to treat, prevent or limit the breakdown of normal tissue at the site of inflammation, and the stable Copper(I) complexes of this invention are effective inhibitors of elastase.

The stable Copper(I) complexes of this invention may also be used in the regulation and/or modulation of lipid metabolism in general. For example, hypercholesterolemia and hyperlipidemia are common and serious health problems which are treatable with the stable Copper(I) complexes of this invention.

Hypercholesterolemia has been observed in marginal and severely copper-deficient rats, as well as other animals, including humans (Lei, "Plasma Cholesterol Response in Copper Deficiency," Role of Copper in Lipid Metabolism, ed. Lei, CRC Press, pages 1-24, 1990). Elevation in serum cholesterol level has been linked to increases in the activity of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase, E.C.1.1.1.34) and glutathione levels (Bunce, "Hypercholesterolemia of Copper Deficiency is Linked to Glutathione Metabolism and Regulation of HMG CoA Reductase," Nutr. Rev. 51: 305-307, 1993; Kim et al., "Inhibition of Elevated Hepatic Glutathione Abolishes Copper Deficiency Cholesterolemia," FASEB J. 6: 2467-2471, 1992).

Similar increases in the synthesis and level of other hepatic lipids (fatty acids, triacylglycerols and phospholipids) have been observed in copper deficient rats

(al-Othman et al., "Copper Deficiency Increases *In Vivo* Hepatic Synthesis of Fatty Acids, Triacylglycerols, and Phospholipids," Proc. Soc. Exp. Biol. Med. 204(1): 97-103, 1993) and treatment with a copper(II) complex has been shown to lower the activity of liver enzymes involved in lipid metabolism, including acetyl CoA synthetase in vivo (Hall et al., "Hypolipidemic Activity of Tetetrakis-mu-(trimethylamine-boranecarboxylato)-bis(trimethylamine-carboxylborane)-dicopper(II) in Rodents and its Effect on Lipid Metabolism," J. Pharm. Sci. 73(7): 973-977, 1984). Conversely, it has been reported that treatment by injection of copper(II) increased serum cholesterol concentrations in rats, possibly by increasing the activity of the HMG CoA reductase (Tanaka et al., "Effect of Cupric Ions on Serum and Liver Cholesterol Metabolism," Lipids 22: 1016-1019, 1987). Accordingly, it is believed that copper may be an important factor in the regulation of lipid levels.

Acetyl CoA synthetase catalyzes the formation of acetyl CoA from acetate. As illustrated in Figure 4, acetyl CoA can be further metabolized along many different pathways leading primarily to the formation of cholesterol and fatty acids or energy production. Agents which inhibit this enzyme influence the biosynthesis of various lipids. HMG-CoA reductase (3-hydroxy-3-methylglutaryl coenzyme A reductase) is located biochemically later in the lipid synthesis scheme and converts HMG-CoA to mevalonic acid, and is the rate limiting reaction in cholesterol biosynthesis (see Figure 4). Stable Copper(I) compounds of this invention inhibit certain key enzymes involved in the formation of lipids, and thus serve as lipid modulating or regulating agents. (The ability of stable Copper(I) complexes to inhibit enzymes in the formation of lipids is disclosed in further detail in Examples 12-13.)

The stable Copper(I) complexes of this invention may also serve as modulating agents of signal transduction in cells. Most intracellular signaling processes are regulated by reversible phosphorylation of specific proteins by kinases. Breakdown of phosphatidylinositol leads to the formation of diacylglycerol and inositol triphosphate, the former acting synergistically with calcium to activate Protein Kinase C (PKC), resulting in translocation of the enzyme from cytosol to the membrane. Phosphorylation of proteins by PKC has been implicated as a pivotal regulatory element in signal transduction, cellular regulation and tumor promotion. Inhibitors of PKC, as well as other protein kinases, have the potential to block proliferative signaling in tumor induction, atherosclerosis and immune modulation.

Examples of factors which stimulate the G-protein linked phospholipase C breakdown of phosphatidylinositol include angiotensin II, bradykinin, endothelin, f-Met-Leu-Phe, and vasopressin. These protein kinase C enzymes are also directly activated by tumor promoters such as phorbol esters. Examples of Receptor linked tyrosine kinases include Epidermal Growth Factor, Nerve Growth Factor, and Platelet Derived Growth Factor. Examples of cytoplasmic tyrosine kinase activators include cytokines such as Interleukin 2, Interleukin 3, and Interleukin 5. These factors bind to specific lymphocyte receptors which activate the cytoplasmic tyrosine kinase.

The action of PKC and protein tyrosine kinase action is illustrated in Figure 5. The stable Copper(I) complexes of this invention serve as signal transduction modulating agents by inhibiting one or more enzymes involved in intracellular signal transduction, including PKC and protein tyrosine kinases.

When administered to an animal to treat the conditions discussed above, the stable Copper(I) complexes may first be combined with one or more suitable carriers

or diluents to yield a pharmaceutical preparation suitable for topical, oral or parenteral application. Such diluents or carriers, however, should not interact with the stable Copper(I) complex to significantly reduce the effectiveness thereof, or oxidize Copper(I). Effective administration will preferably deliver a dosage of approximately 0.01 to 100 mg of the stable Copper(I) complex per kg of body weight.

Methods for encapsulating compositions (such as in a coating of hard gelatin) for oral administration are well known in the art (see, e.g., Baker, Richard, Controlled Release of Biological Active Agents, John Wiley and Sons, 1986)(incorporated herein by reference). Suitable carriers for parenteral application (such as intravenous, subcutaneous or intramuscular injection) include sterile water, physiological saline, bacteriostatic saline (saline containing 0.9 mg/ml benzyl alcohol) and phosphate-buffered saline. The stable Copper(I) complexes may be topically applied in the form of liquids, containing pharmaceutically acceptable diluents (such as saline and sterile water) or may be applied as lotions, creams or gels, containing additional ingredients to impart the desired texture, consistency, viscosity and appearance. Such additional ingredients are familiar to those skilled in the art and include emulsifying agents such as non-ionic ethoxylated and nonethoxylated surfactants, fatty alcohols, fatty acids, organic or inorganic bases, preserving agents, wax esters, steroid alcohols, triglyceride esters, phospholipids such as lecithin and cephalin, polyhydric alcohol esters, fatty alcohol esters, hydrophilic lanolin derivatives, hydrophilic beeswax derivatives, hydrocarbon oils such as palm oil, coconut oil, mineral oil, cocoa butter waxes, silicon oils, pH balancers and cellulose derivatives.

Topical administration may by accomplished by applying an amount of the preparation directly to the

desired area, such as a wound or an inflamed area. The required dosage will vary according to the particular condition to be treated, the severity of the condition, and the duration of the treatment. Preferably, when the stable Copper(I) complex is topically applied in the form of a lotion, cream or gel, the preparation may contain about 1% to about 20% of a penetration enhancing agent. Examples of penetration enhancing agents include dimethylsulfoxide (DMSO), urea and eucalyptol. In the case of a liquid preparations for topical application, the concentration of penetration enhancing agent (such as DMSO) may comprise about 30% to about 80% of the preparation.

In addition to the activity discussed above, the stable Copper(I) complexes of this invention also possess utility as hair growth agents. Hair loss is a common affliction of humans, the most common being "alopecia" where males lose scalp hair as they get older (also called "male pattern baldness"). Other hair loss afflications include alopecia areata (AA), female pattern baldness and secondary alopecia (e.g., hair loss associated with chemotherapy and/or radiation treatment). The stable Copper(I) complexes of this invention are particularly useful in stimulating hair growth associated with any hair loss afflication, including the specific afflications identified above.

Hair is normally divided into two types, "terminal" and "vellus" hairs. Terminal hair is coarse, pigmented hair which arises from follicles which are developed deep within the dermis. Vellus hairs are typically thin, non-pigmented hairs which grow from hair follicles which are smaller and located superficially in the dermis. As alopecia progresses, there is a change from terminal to vellus type hair. Other changes that contribute to alopecia are alterations in the growth cycle of hair. Hair typically progresses through three cycles, anagen

(active hair growth), catagen (transition phase), and telogen (resting phase during which the hair shaft is shed prior to new growth). As baldness progresses, there is a shift in the percentages of hair follicles in each phase, with the majority shifting from anagen to telogen. The size of hair follicles is also known to decrease while the total number remains relatively constant.

As mentioned above, the stable Copper(I) complexes of this invention have utility as stimulating agents for the growth of hair in warm-blooded animals. In one embodiment of the present invention, the Copper(I) complex may be administered intradermally in the area to be treated, along with a suitable vehicle, at a concentration of approximately 100-500 micrograms of Copper(I) complex per 0.1 ml of vehicle. Suitable vehicles in this regard include saline, sterile water, and the like.

In another embodiment, the stable Copper(I) complex may be topically applied in the form of a liquid, lotion, cream or gel by applying an effective amount of the topical preparation directly to the scalp. Any quantity sufficient to stimulate the rate of hair growth is effective, and treatment may be repeated as often as the progress of hair growth indicates. Preferably, suitable topical hair growth preparations contain from about 0.1% to about 20% by weight of the stable Copper(I) complex (based on the total weight of the preparation).

Topical hair growth preparations of the present invention may contain about 0.5% to about 10% of an emulsifying or surface active agent. Non-ionic surface active agents and ionic surface active agents may be used for the purposes of the present invention. Examples of suitable non-ionic surface active agents are nonylphenoxypolyethoxy ethanol (Nonoxynol-9), polyoxyethylene oleyl ether (Brij-97), various polyoxyethylene ethers (Tritons), and block copolymers of ethylene oxide and propylene oxide of various molecular

weights (Pluronic 68, for example). Acceptable preparations may also contain about 1% to about 10% of certain ionic surface active agents. These ionic surface active agents may be used in addition to or in place of, the non-ionic surface active agents. Examples of ionic surface active agents are sodium lauryl sulfate and similar compounds.

In addition to, or in place of, the emulsifying or surface active agent, topical hair growth preparations of this invention may contain about 1% to about 20% of a penetration enhancing agent. Examples of penetrating enhancing agents are DMSO and Urea. In the case of a liquid preparation to be applied topically, the concentration of a penetrating enhancing agent, such as DMSO, may comprise about 30% to about 80% of the topical preparation. The balance of the topical hair growth preparation may comprise an inert, physiologically acceptable carrier. Suitable carriers include, but are not limited to, water, physiological saline, bacteriostatic saline (saline containing 0.9 mg/ml benzyl alcohol), petrolatum based creams (e.g., USP hydrophilic ointments and similar creams, Unibase, Parke-Davis), various types of pharmaceutically acceptable gels, and short chain alcohols and glycols (e.g., ethyl alcohol and propylene glycol).

The following are examples of suitable hair growth preparations within the context of the present invention:

Preparation A:

Copper(I) Complex	10.0%	(w/w)
Hydroxy Ethyl Cellulose	3.0%	
Propylene Glycol	20.0%	
Nonoxynol-9	3.0%	
Sodium Lauryl Sulfate	2.0%	
Benzyl Alcohol	2.0%	
0.2N Phosphate Buffer	60.0%	

Preparation B:

Copper(I) Complex	10.0%	(w/w)
Nonoxynol-9	3.0%	
Ethyl Alcohol	87.0%	

Preparation C:

Copper(I) Complex	5.0%	(w/v)
Ethyl Alcohol	47.5%	
Isopropyl Alcohol	4.0%	
Propylene Glycol	20.0%	
Laoneth-4	1.0%	
Water	22.5%	

Preparation D:

Copper(I) Complex	5.0%	(w/v)
Water	95.0%	

Preparation E:

Copper(I) Complex	5.0%	(w/v)
Hydroxypropyl Cellulose	2.0%	
Glycerin	20.0%	
Nonoxynol-9	3.0%	
Water	70.0%	

Preparation F:

Copper(I) Complex	1.0%	(w/w)
Nonoxynol-9	5.0%	
Unibase Cream	94.0%	

Preparation G:

Copper(I) Complex	2.0%	(w/w)
Nonoxynol-9	3.0%	
Propylene Glycol	50.0%	
Ethanol	30.0%	
Water	15.0%	

The Copper(I) complexes of the present invention also posses utility as anti-viral agents, and are particularly effective in the inhibition of the AIDS virus. Human acquired immunodeficiency syndrome or "AIDS" is a fatal disease for which there is presently no cure. The disease

is believed to be caused by a virus known as the human immunodeficiency virus, commonly referred to as "HIV." The virus is transmitted by HIV-infected individuals through the exchange of bodily fluids. HIV infection results most commonly from sexual contact with an infected partner and the sharing among intravenous drug users of hypodermic syringes previously used by an infected individual. A pregnant HIV-infected mother may infect her unborn child by trans-placental transmission, and HIV-contaminated blood is a possible source of infection for individuals subject to blood transfusion.

HIV infection causes a suppression of the immune system. The immune suppression renders the infected individual vulnerable to a variety of opportunistic infections and conditions that are otherwise kept in balance by a healthy immune system. Fatalities result from HIV infection due to the inability of AIDS patients to respond to treatment of the opportunistic infections and conditions as a consequence of their compromised immune systems. Because the virus may often remain dormant, the manifestation of AIDS from HIV infection may take as long as ten years.

One approach to the treatment of AIDS has targeted the opportunistic infections or conditions which result from HIV infection. The treatment of such infections or conditions, however, is ultimately ineffective and, while prolonging the life of the infected individual, does not treat the underlying HIV infection. A second approach to the treatment of AIDS targets the cause of the disease itself. Because AIDS results from viral infection, it is believed that viral inactivation may ultimately provide a cure. Materials which are capable of viral inactivation or inhibition are referred to herein as "antiviral agents."

To understand the mode of action of antiviral agents in the treatment of AIDS, an understanding of the process

of HIV infection is necessary. HIV chronically infects specific immune cells known as T-helper cells, which are required for normal immune response. The HIV infected T-helper cells serve as hosts to the virus and facilitate the reproduction of the virus (the process of viral reproduction is commonly referred to as "replication"). After HIV infection, the infected host cell eventually dies, the replicated HIV virus is released, and the infection spreads to additional cells. This cycle continues unabated, depleting the population of T-helper cells and, in time, weakens the immune system to the onset of AIDS symptoms. Because T-helper cells are continuously produced by the body, the population of these cells may be reestablished in the absence of further HIV infection. Therefore, the progression of HIV infection (and the subsequent onset of AIDS) may be arrested by the prevention or inhibition of viral replication, and antiviral agents capable of inhibiting or preventing the replication of HIV should be effective in the treatment of AIDS.

At the genetic level, HIV replication requires the insertion of viral deoxyribonucleic acid ("DNA") into the genome of the host cell. The genome of the host cell consists of the cell's own DNA, and is responsible for the synthesis of materials essential to the cell's own function and proliferation. Once the viral DNA is inserted into the host genome, the host facilitates replication of HIV. The inserted viral DNA is an enzymatic product derived from viral ribonucleic acid ("RNA") and the action of an enzyme known as HIV reverse transcriptase. Inhibition of HIV reverse transcriptase precludes the formation of viral DNA required for insertion into the genome of the host. Viral replication is prevented by the absence of viral DNA in the host cell genome. Antiviral agents which inhibit HIV reverse

transcriptase are thus potential therapeutic drugs for treatment of AIDS.

Accordingly, in yet another embodiment of the present invention, antiviral agents are disclosed for inhibiting HIV replication, as well as methods relating to the administration thereof to an HIV-infected patient. The antiviral agents of this invention are the stable Copper(I) complexes discloses above, and the methods include administration of a therapeutically effective amount of a composition which includes a stable Copper(I) complex in combination with a pharmaceutically acceptable carrier or diluent. Although not limited by the following theory, it is believed that the Copper(I) complexes of this invention enhance transport of Copper(I) into HIV infected cells which, in turn, inhibits or inactivates HIV protease and thus inhibits the replication of HIV. As used herein, the term "HIV" includes the various strains of the virus such as HIV-1 and HIV-2.

Administration of the stable Copper(I) complexes of the present invention may be accomplished in any manner which will result in a systemic dose of a therapeutically effective amount of the Copper(I) complex to an HIV-infected animal or patient (including human patients). For example, such administration may be by injection (intramuscular, intravenous, subcutaneous or intradermal), oral, nasal, or suppository applications. Typically, preparations of the present invention include stable Copper(I) complexes in solution for various forms of injection, or in preparations which are formulated for the sustained release of the stable Copper(I) complexes for oral, nasal, or suppository dosage application and generally include one or more inert, physiological acceptable carriers. As used herein, the term "effective amount" means an amount of the stable Copper(I) complex which inhibits HIV replication in the patient. Suitable

dosages may range from approximately 0.01 to 100 mg of stable Copper(I) complex per kg body weight.

The stable Copper(I) complexes of this invention may be screened for their ability to inhibit HIV replication using known techniques. For example, HIV virus replication may be monitored using the Cytopathic Effect (CPE) assay disclosed by Bergeron et al. (J. Virol. 66:5777-5787, 1992). In this assay, the degree of infection is monitored by the appearance of fused cellular membranes ("syncitium"). Alternatively, assays directed to activity of HIV protease may be employed. For example, the assays and techniques disclosed in the following references may be employed: Ashorn et al., Proc. Natl. Acad. Sci. U.S.A. 87:7472-7476, 1990; Schramm et al., Biochem. Biophys. Res. Commun. 179:847-851, 1991; Sham et al., Biochem. Biophys. Res. Commun. 175:914-919, 1991; and Roberts et al., Science 248:358-361, 1990. Moreover, the ability of the stable Copper(I) complexes of this invention to inhibit HIV replication may be determined by the assay disclosed in Example 5 herein below.

Inhibition of viral replication by the stable Copper(I) complexes of this invention may also be due to inhibition and/or prevention of viral entry into a cell. With respect to HIV, for example, the stable Copper(I) compounds are believed to prevent viral entry by interfering with CD4 receptor binding and membrane fusion. This is illustrated in Example 20 which presents data directed to the effect of a stable Copper(I) complex of this invention on syncytium formation using a virus-free, genetically engineered syncytium formation assay (Fu et al., J. Virol 7:3818, 1993). This assay relies upon the molecular recognition of gp120, gp41 and the CD4 receptor to create syncytium, and representative stable Copper(I) complexes of this invention were found to inhibit syncytium formation. This indicates that the stable Copper(I) complexes inhibited HIV replication by

preventing viral entry, presumably by interacting with the viral proteins gp120 and gp41, and thus prevented and/or inhibited gp120 and gp41 function related to viral binding and membrane fusion. These results provide evidence that the stable Copper(I) complexes of the present invention have utility in preventing and/or inhibiting the spread of HIV to uninfected cells.

Accordingly, in this aspect of the present invention, stable Copper(I) complexes may be formulated in a manner suitable for application to, for example, the vaginal or rectal mucosa, as well as the penis. Suitable formulations include, but are not limited to, solutions, creams, gels, ointments, foams, suppositories and powders, and may include a variety of additional components such as lubricants, preservatives, carriers and diluents, as well as other active ingredients such as spermacides. Such formulations contain a sufficient quantity of the stable Copper(I) complex, and are applied to the epithelium of the vaginal mucosa, cervix, anus and/or penis in an amount sufficient to prevent and/or inhibit viral transmission.

In this embodiment, the stable Copper(I) complexes of the present invention may also serve to prevent and/or inhibit the transmission of sexually transmitted diseases in addition to HIV, including human herpes virus and Hepititis virus (as well as Chlamydia). The stable Copper(I) complexes of this invention may also have contraceptive activity.

As mentioned above, the stable Copper(I) complexes of this invention, in addition to inhibiting HIV replication, may also inhibit replication of other viruses. Such viruses include, but are not limited to, human T-cell leukemia (HTLV) I and/or II, human herpes virus (HSV1 and 2), cytomegalo virus (human, hCMV, and murine, mCMV), encephalomyocarditis viruses (HAV, HBV, HCV (EMCV), Epstein Barr virus (EBV), human hepatitis virus (e.g., hepatitis B virus, HBV), Varicella Zoster virus,

Rhinovirus, rubella virus, respiratory syncytium virus (RSV), influenza viruses A and B, parainfluenza viruses and adenovirus. One skilled in the art could readily assay the stable Copper(I) complexes of this invention for their inhibitory activity with regard to these viruses, as well as other viruses. For example, Example 15 illustrates the inhibitory affect of stable Copper(I) complexes of this invention on both encephalomyocarditis virus (EMCV) and cytomegalo virus (CMV). Furthermore, Example 21 illustrates the inhibitory activity of stable Copper(I) complexes against HIV-1_{LAV}, as well as against HIV-2_{ROD2}, SIV_{SMN} and HIV-1_{FTC^R} (an FTC resistant strain of HIV-1 which is not resistant to AZT).

In addition to the biological activity of the stable Copper(I) complexes of the present invention, the multi-dentate ligands of this invention also possess biological activity when administered alone as the "free" multi-dentate ligand (i.e., without Copper(I)). Such biological activity includes the activities identified above, including anti-viral activity, as well as a preventative agent against gastric tissue damage. Although not intending to be limited to the following theory, when the multi-dentate ligands of this invention are administered as the free ligand, it is believed that they function, at least in part, by scavenging Copper(I) to yield the stable Copper(I) complex in vivo.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

The examples which follow illustrate the preparation and utility of certain exemplary embodiments of the stable Copper(I) complexes of the present invention. To summarize the examples that follow: Example 1 illustrates

the synthesis of neocuproine Copper(I) at a molar ratio of 1:1 and 2:1; Example 2 illustrates the superoxide dismutase (SOD)-mimetic activity of representative Copper(I) complexes of this invention (employing a copper(II)-peptide complex as a positive control); Example 3 illustrates the wound healing activity of a representative Copper(I) complex of this invention; Example 4 illustrates hair growth activity of a representative Copper(I) complex of this invention; Example 5 illustrates inhibition of HIV replication by a representative Copper(I) complex of this invention; Example 6 illustrates the activity of a representative "free" multi-dentate ligand of this invention for both wound healing and protection against ethanol-induced gastric mucosal damage; Examples 7 and 8 illustrates the inhibition of cyclooxygenase-1 and cyclooxygenase-2, respectively, by representative stable Copper(I) complexes; Example 9 illustrates the inhibition of 5-lipoxygenase by representative stable Copper(I) complexes; Example 10 illustrates the inhibition of leukotriene C_4 synthetase by representative stable Copper(I) complexes; Example 11 illustrates the inhibition of elastase by a representative stable Copper(I) complex; Example 12 illustrates the inhibition of acetyl coenzyme A synthetase by representative stable Copper(I) complexes; Example 13 illustrates the inhibition of HMG-CoA reductase by representative stable Copper(I) complexes; Example 14 illustrates the inhibition of HIV-1 activity by various isomers of a representative stable Copper(I) complex; Example 15 illustrates the anti-viral activity of representative stable Copper(I) complexes and a representative free multi-dentate ligand; Example 16 illustrates inhibition of HIV-1 and HIV-2 proteases by representative stable Copper(I) complexes; Example 17 illustrates the inhibition of HIV reverse transcriptase by representative stable Copper(I) complexes; Examples 18 and

19 illustrate the inhibition of Protein Kinase C and various tyrosine kinases, respectively, by representative stable Copper(I) complexes; Example 20 illustrates inhibition of syncytium formation by representative stable Copper(I) complexes using a virus-free, genetically engineered syncytium assay (Fu et al., J. Virol 7:3818, 1993); Example 21 illustrates the inhibitory activity of representative stable Copper(I) complexes against several different virus strains; and Example 22 illustrates inhibitory activity of representative stable Copper(I) complexes against pathological human viruses.

Example 1
Synthesis of Copper(I)-Neocuproine

Neocuproine hydrate was used as received from Aldrich Chemical Company, having the following properties: mp161-163°C; ^{1}H NMR (500MHz, DMSO-d_6) δ 8.32 (2H, d, J = 8.2), 7.85 (2H, s), 7.60 (2H, d, J = 8.1), 2.79 (6H, s); ^{13}C NMR (125MHz, DMSO-d_6) δ 158.0, 144.6, 136.1, 126.4, 125.3, 123.1, 24.9.

A. Neocuproine Copper(I) (1:1)

Cuprous chloride (1.98g, 20.0mmol) was added to a stirred, vacuum-degassed solution of neocuproine hydrate (4.53g, 20.0mmol) in acetonitrile (150mL). This solution was stirred for 2 hours. The resulting suspension was warmed to boiling and filtered. The filtrate was boiled to a volume of about 100mL. This solution was allowed to cool slowly to give dark red needles: mp280-284°C(decomp., lit. 310-320°C); UV-vis λmax (CH_2Cl_2) 232 nm (ε=109,000M^{-1} cm^{-1}), 275nm (ε=85,500), 454nm (ε=4,970),(Healy et al., J. Chem. Soc. Dalton Trans. 2531, 1985); ^{1}H NMR (500MHz, DMSO-d_6) δ 8.74 (2H, d, J = 8.2), 8.21 (2H, s), 7.95 (2H, d, J = 8.2), 2.38 (6H, s); ^{13}C NMR (125MHz, DMSO-d_6) δ 157.6, 142.2, 137.4, 127.1, 125.9, 125.6, 25.1; Anal.

calcd. for $C_{14}H_{12}ClCuN_2$: C, 54.73; H, 3.94; N, 9.12; Cl, 11.54. Found: C, 54.67; H, 3.89; N, 9.04; Cl, 11.40.

B. Neocuproine Copper(I) (2:1)

A vacuum degassed solution of neocuproine hydrate (4.53g, 20.0mmol) in absolute ethanol (150mL) was added to cuprous chloride (990mg, 10.0mmol) via cannula under an atmosphere of nitrogen. The resulting bright red solution was stirred at room temperature for 2 hours. This mixture was filtered, to remove a small amount of insoluble matter, and evaporated to give 5.64g (100%) of bright red solid. Recrystallization from aqueous methanol gave very fine needles: mp231-233°C; UV-vis λ_{max} (95% ethanol) 207nm (ε = 63,750$M^{-1}cm^{-1}$), 226nm (ε = 76,250), 272nm (ε = 60,000), 454nm (ε = 6,750), ^1H NMR (500MHz, DMSO-d_6) δ 8.75 (2H, br s), 8.22 (2H, s), 7.96 (2H, br s), 2.40 (6H, s); ^{13}C NMR (125MHz, DMSO-d_6) δ 157.6, 142.2, 137.3, 127.1, 125.8, 125.6, 25.0; Anal. calcd. for $C_{28}H_{24}ClCuN_4$: C, 65.24; H, 4.69; N, 10.87; Cl, 6.88; Cu, 12.33. Found: C, 65.01; H, 4.73; N, 10.75; Cl, 6.84; Cu, 12.70.

Example 2
Superoxide Dismutase Mimetic Activity of Copper(I) Complex

As used herein, compounds which possess activity in a superoxide dismutase (SOD) assay are termed "SOD mimetics." In this example, representative Copper(I) complexes of this invention were evaluated for SOD mimetic activity as measured by the Xanthine Oxidase/NBT method (see Oberly and Spitz, Handbook of Methods for Oxygen Radical Research, R. Greenwald (ed.), pp. 217-220, 1985; Auclair and Voisin, Handbook of Methods for Oxygen Radical Research, R. Greenwald (ed.), pp. 123-132, 1985). The reactions contained the following: 100 µM Xanthine, 56 µM NBT (Nitro Blue Tetrazolium), 1 unit of Catalase, 50 mM

Potassium Phosphate Buffer, pH 7.8. The reaction was initiated by the addition of Xanthine Oxidase in sufficient quantity to obtain an increase in absorbance at 560 nm of approximately 0.025/min. in a total volume of 1.7 ml. The Xanthine Oxidase was prepared fresh daily and stored on ice until used. All the components of the reaction are added except the Xanthine Oxidase and the spectrophotometer was adjusted to zero at 560 nm. The reaction was initiated by the addition of the Xanthine Oxidase. All reagents were obtained from Sigma Chemical Co.

Measurements of the Absorbance at 560 nm were taken at 1-2 minute intervals for at least 16 minutes. The control consisted of reactions containing zero Copper(I) complex. The Copper(I) complexes tested in this example were as follows: bathocuproine disulfonate Copper(I) ("BCDS:Cu(I)"); neocuproine Copper(I) ("NC:Cu(I)"); and 2,2'-biquinoline Copper(I) ("BQ:Cu(I)"). As a positive control, reactions containing a peptide-copper(II) complex (i.e., glycyl-L-histidyl-L-lysine:copper(II) or "GHK:Cu"), which is a known SOD mimetic (see U.S. Patent No. 4,760,051), were also employed. One unit of SOD activity was taken as that amount of sample in micromoles which inhibits the control reaction with the NBT by 50%. The relative activity is then obtained by comparing the micromoles of Copper(I) complex necessary to product a 50% inhibition of the control reactions. The lower the value, the more active the compound is as an SOD mimetic. The results of this experiment are presented in Table 8 below.

Table 8
SOD-Mimetic Activity of Copper(I) Complexes

Exp. No.	Compound	Copper Ratio (ligand:Copper)	Activity (µmol per ½Max. Inhib.)	Relative Activity to Control
1	GHK:Cu(II)	2:1	0.055	---
	BCDS:Cu(I)	2:1	0.034	1.6
2	GHK:Cu(II)	2:1	0.0503	---
	BCDS:Cu(I)	2:1	0.0278	1.8
	BCDS:Cu(I)	2:1	0.0018	28
	NC:Cu(I)	2:1	0.0014	36
3	GHK:Cu(II)	2:1	0.0479	---
	NC:Cu(I)	1:1	0.0018	27
	BQ:Cu(I)	2:1	0.0028	17

Example 3
Wound Healing Activity
of Copper(I) Complexes

The subcutaneous implantation of stainless steel wound chambers in rats provides a model for the healing of open cavity wounds. Implantation of these chambers triggers a series of responses which reflect the series of phases involved in wound healing - fibrin clot formation, infiltration of white cells, collagen synthesis, and new blood vessel formation.

This assay involves the implantation of a stainless steel chamber (1 X 2.5 cm cylindrical 312 SS, 20 mesh, with Teflon end caps) on the dorsal mid-line of rats. After one week to allow for encapsulation of the chamber, the chamber on each rat was injected with a 0.2 ml saline

solution containing 2.7 µmol of the Copper(I) complex (i.e., BCDS Copper(I) 1:1 or 2:1), or with the same volume of saline (0.2 ml) without the Copper(I) complex (i.e., control). Injections were made on days 5, 7, 9, 12, 14, 16 and 19. The chambers were then removed on day 21.

The chambers were lyophilized and the interior contents removed for biochemical analysis. The biochemical parameters examined included the total dry weight, protein content, collagen content (i.e., hydroxyproline content after acid hydrolysis) and glycosaminoglycan content or "GAG" (i.e., uronic acid content after acid hydrolysis).

The protein was determined by the method of Lowry et al. (J. Biol. Chem. 193: 265-275, 1951) using Bovine Serum Albumin (BSA) as a standard. The collagen content was determined by acid hydrolysis and a colorimetric assay for hydroxyproline (Bergman et al., Clin. Chim. Acta 27:347-349, 1970), an amino acid specific for collagen. Glycosaminoglycan content was determined by quantitation of the amount of uronic acid (UA). Aliquots of the homogenate were dissolved in 0.5M NaOH, precipitated and washed with ethanol, and uronic acid was determined by a colorimetric assay using 2-phenylphenol as a reagent (Vilím V., Biomed. Biochem. Acta. 44(11/12s):1717-1720, 1985). Glycosaminoglycan content was expressed as µg of uronic acid per chamber.

The results of this experiment are illustrated in Figure 1. Specifically, BCDS Copper(I) at both the 1:1 and 2:1 ratio significantly stimulated the glycosaminoglycan content of the injected chamber. Moreover, BCDS Copper(I) at both ratios stimulated the collagen content of the injected chambers. Collagen and glycosaminoglycans are two of the critical extracellular matrix components important for tissue regeneration associated with wound healing.

Example 4

Stimulation of Hair Growth by Copper(I) Complexes

The following example illustrates the stimulation of hair growth in warm-blooded animals after intradermal injection of a Copper(I) complex of this invention.

The backs of C3H mice (60 days old, telogen hair growth phase) were closely clipped on day 1 using an electric clipper. A sterile saline solution containing the indicated copper complex was then injected intradermally (i.e., infiltrated under the skin) at two locations within the clipped areas of the mice. Injection at two locations provided two test locations within the clipped area of each mouse. Each injection (0.1 ml) contained the indicated amount of the Copper(I) complex (i.e., BCDS Copper(I) (1:1) complex at 0.14 µmol and 1.4 µmol) within a sterile saline solution. A group of saline injected mice (0.1 ml) served as controls. Following injection of the Copper(I) complex, indications of hair growth were seen within 10 days. The first visual signs were a darkening of the skin in a circular region surrounding the injection site. The size of this region is generally dose dependent, increasing with an increase in dose. The 0.1 ml injections used in this experiment produced a circle of hair growth measuring approximately 0.5 cm^2 to 5 cm^2 in diameter. Active hair growth occurred between 14-20 days following injection, with a maximum effect seen by day 29. Both the number of mice growing hair at the injection site and the diameter of the hair growth region were determined at day 21. A positive response was expressed as the number of mice exhibiting hair growth at the injection sites compared to the total number of mice injected in the study. The results of this experiment are presented in Table 9 below.

Table 9
Hair Growth Activity of BCDS Copper(I) Complex

Amount Injected (μmol)	Growth Area (cm^2)
0.0 (control)	0.0
0.14	1.35 (Std. Dev. 0.42)
1.4	3.06 (Std. Dev. 0.47)

Example 5
Inhibition of HIV Replication of Copper(I) Complex

In this experiment, the inhibitory effect of bathocuproine disulfonic acid (BCDS) Copper(I) (2:1) complex on phytohemagglutinin (PHA) stimulated peripheral blood mononuclear cells is demonstrated.

PHA stimulated peripheral blood mononuclear cells (PBMC) were infected by HIV_{IIIB} in the presence of the Copper(I) complex identified above and cultured in the presence of the Copper(I) complex for two weeks. The extent of HIV replication was assayed at 1 and 2 weeks by a p24 antigen capture ELISA assay. More specifically, PBMC was stimulated with PHA for 24 to 72 hours in basal medium, containing RPMI-1640, 10% fetal bovine serum, and 50 μg/mL gentamicin, and then cultured overnight in the presence of 250 units/ml IL-2. Treated PBMC were pelleted by centrifugation and resuspended to 0.75 x 10^6/mL in basal medium with appropriate dilutions of the Copper(I) complex or with no Copper(I) complex added (i.e., control). To each 0.5 mL aliquot of cells, 0.5 mL of appropriate HIV dilution was added. The virus-cell mixture was incubated for 2 hours at 37°C in a 5% CO_2 humidified atmosphere. Following the incubation period, the PBMC were washed twice in phosphate-buffered saline. Cells were resuspended in 5 mL to 7 x 10^4 cells/mL in basal medium with (or without) the Copper(I) complex. Each cell aliquot was dispensed into four replicate wells of a 48

well tissue culture plate. Cells were fed twice a week with appropriate medium.

At one week and two week culture timepoints the extent of HIV replication was assayed by a p24 antigen capture assay kit (Coulter Corp., Hialeah, Florida). PBMC were treated with buffered detergent to release viral proteins. The cell extract was absorbed to immunoassay titer plates and p24 was detected by binding of a monoclonal anti-p24 antibody coupled to an enzyme. Following the addition of a chromogenic substrate, the amount of p24 was quantified spectrophotometrically.

The results of this experiment are presented in Figure 2. In particular, a 50 μM concentration of the BCDS Copper(I) (2:1) complex completely inhibited HIV replication at both week 1 and week 2 at the identified virus dilutions. Furthermore, the 5 μM concentration of BCDS Copper(I) (2:1) complex completely inhibited HIV replication at week 2 at the 10^{-6} virus dilution.

Example 6
Activity of "Free" Multi-Dentate Ligand

This example illustrates the activity of the free multi-dentate ligands of this invention. As used herein, the free ligand is not complexed to the Copper(I) ion prior to administration.

A. Inhibition of Ethanol-Induced Gastric Mucosal Damage

Juvenile Sprague-Dawley rats were used in this example. After fasting for 24 hours, the rats were treated by oral gavage with bathocuproine disulfonic acid (BCDS) as the Copper(I)-free ligand at various dosages (i.e., 0, 7.6 and 37.6 mg/kg body weight). One hour after BCDS treatment, the animals were challenged with 1 ml of 95% ethanol by oral gavage to cause erosion of the gastric

mucosa. As shown in Table 10, BCDS pre-treatment led to a dose-dependent protection against the mucosal damage observed in the control animals.

Table 10
Effect of BCDS on Ethanol-Induced
Gastric Mucosal Damage

Dosage mg/kg body weight	Mucosal Damage % of total area	
	Mean	S.E.M.
0.0	45.48	6.94
7.6	32.95	7.49
37.6	23.45	8.18

B. Wound Healing Activity

The BCDS ligand was also examined in the rat wound chamber model as disclosed above in Example 3. The results of this experiment are presented in Table 11.

Table 11
Effect of BCDS on Wound Healing

mg/injection	ug uronic acid/mg protein
0.0 (control)	28.3 (Std. Dev. ± 8.7)
1.5	57.6 (Std. Dev. ± 9.1)
7.5	79.2 (Std. Dev. ± 10.8)

These results indicate that glycosaminoglycan synthesis is stimulated by administration of the free BCDS ligand.

Example 7
Inhibition of Cyclooxygenase-1 by Neocuproine and BCDS Copper(I) Complexes (2:1)

Cyclooxygenase is involved in the formation of prostaglandins and thromboxanes by the oxidative metabolism of arachidonic acid (see Figure 3).

In this series of experiments, cyclooxygenase-1 from ram seminal vesicles was incubated with arachidonic acid (100 μM) for 2 minutes at 37° C in the presence of neocuproine Copper(I) (2:1) or BCDS Copper(I) (2:1) at increasing concentrations of neocuproine Copper(I) or BCDS Copper(I) from 0.3 to 300 μM (the control consisted of reactions in the absence of the stable Copper(I) complex). The assay was terminated by the addition of trichloroacetic acid (TCA), and cyclooxygenase-1 activity was determined by reading the absorbance at 530 nm (Evans et al., "Actions of Cannabis Constituents on Enzymes of Arachidonate Metabolism:Anti-inflammatory Potential," Biochem. Pharmacol. 36: 2035-2037, 1987; Boopathy and Balasubramanian, "Purification and Characterization of Sheep Platelet cyclooxygenase," Biochem J. 239: 371-377, 1988).

Neocuproine Copper(I) (2:1) was found to inhibit cyclooxygenase-1 with an IC50 of 23μM (*see* Table 12). BCDS Copper(I) (2:1) complex produced approximately 44% inhibition at a concentration of 300 μM. These results demonstrate that the stable Copper(I) complexes of this invention are potent inhibitors of prostaglandin synthesis through inhibition of cyclooxygenase-1.

Table 12
Inhibition of Cyclooxygenase-1 by
Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	300	43.5 ± 1.5
Neocuproine Copper(I) (2:1)	300	77.3 ± 1.5
	30	54.5 ± 0.5
	3.0	15.5 ± 2.5
	0.3	6.5 ± 0.5

A similar method was used to determine the inhibition of Cyclooxygenase-1 by other stable Copper(I) complexes. The results of these assays are shown below in Table 12a.

Table 12a.
Inhibition of cyclooxygenase-1
by stable Copper(I) complexes

Copper(I) Complex	Cyclooxygenase-1 Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	>>300
Neocuproine Copper(I) (2:1)	23
Biquinoline Copper(I) (2:1)	270
Hexocuproine* Copper(I) (2:1)	210
t-Butylcuproine**-Copper(I) (1:1)	67

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

Example 8

Inhibition of Cyclooxygenase-2 by Neocuproine and BCDS Copper(I) Complexes (2:1)

Cyclooxygenase-2, also known as prostaglandin H synthase-2, catalyzes the oxygenation of unesterified precursors to form cyclic endoperoxide derivatives, including prostaglandin H (see Figure 3).

In this series of experiments, cyclooxygenase-2 from sheep placenta, 80 units/tube, was pre-incubated with 1 mM glutathione (GSH), 1 mM hydroquinone, 2.5 μM hemoglobin, and either neocuproine Copper(I) (2:1) or BCDS Copper(I) (2:1) at increasing concentrations of neocuproine Copper(I) or BCDS Copper(I) from 0.3 to 300 μM for 1 minute at 25°C. The reaction was initiated by the addition of arachidonic acid (100 μM), and terminated after 20 minutes at 37° C by the addition of TCA. Following centrifugal separation of the precipitated protein, thiobarbiturate was added and cyclooxygenase activity was determined by absorbance at 530 nm (see Evans et al., supra; Boopathy and Balasubramanian, supra; O'Sullivan et al., "Lipopolysaccharide Induces Prostaglandin H Synthese-2 in Alveolar Macrophages," Biochem. Biophys. Res. Commun. 187:1123-1127, 1992).

Neocuproine Copper(I) (2:1) was found to inhibit cyclooxygenase-2 at an estimated IC50 of 25μM (*see* Table 13), which is similar to the results of Example 7 with cyclooxygenase-1. BCDS Copper(I) (2:1) produced approximately 34% inhibition at the screening concentration of 300 μM. These results show that stable Copper(I) complexes of this invention are also potent inhibitors of prostaglandin synthesis through inhibition of cyclooxygenase-2.

Table 13
Inhibition of Cyclooxygenase-2 by
Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	300	34.0 ± 1.0
Neocuproine Copper(I) (2:1)	300	63.8 ± 0.5
	30	54.0 ± 1.0
	3.0	7.0 ± 1.0
	0.3	6.5 ± 2.5

A similar method was used to determine the inhibition of Cyclooxygenase-2 by other stable Copper(I) complexes. The results of these assays are shown below in Table 13a.

Table 13a.
Inhibition of Cyclooxygenase-2
by Stable Copper(I) Complexes

Copper(I) Complex	Cyclooxygenase-2 Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	>>300
Neocuproine Copper(I) (2:1)	25
Biquinoline Copper(I) (2:1)	103
Hexocuproine* Copper(I) (2:1)	>>300
t-Butylcuproine** Copper(I) (1:1)	>>300

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

Example 9
Inhibition of 5-Lipoxygenase by Neocuproine and BCDS Copper(I) Complexes (2:1)

The 5-lipoxygenase is the principal lipoxygenase in basophils, polymorphonuclear (PMN) leukocytes, macrophages, mast cells, and any organ undergoing an inflammatory response. As illustrated in Figure 3, the action of 5-lipoxygenase leads to the formation of 5-HPETE and 5-HETE, which are precursors to the leuokotriene LTB_4 and LTC_4.

In this series of experiments, 5-lipoxygenase assays were run using a crude enzyme preparation prepared from rat basophilic leukemia cells (RBL-1). Neocuproine Copper(I) (2:1) or BCDS Copper(I) (2:1) at increasing concentrations from 0.3 to 300 μM were pre-incubated with the 5-lipoxygenase for 5 minutes at room temperature, and the reaction was initiated by addition of arachidonic acid substrate. After incubation at room temperature for 8 minutes, the reaction was terminated by the addition of citric acid. The levels of 5-HETE were determined by a specific 5-HETE RIA (Shimuzu et al., "Enzyme with Dual Lipoxygenase Activities Catalyzes Leukotriene A4 Synthesis from Arachidonic Acid," Proc. Natl. Acad. Sci. U.S.A. 81:689-693, 1984; Egan and Gale, "Inhibition of Mammalian 5-Lipoxygenase by Aromatic Disulfides," J. Biol. Chem. 260:11554-11559, 1985).

Both BCDS Copper(I) (2:1) and neocuproine copper (I) (2:1) were found to be inhibitors of 5-lipoxygenase with estimated IC50's of less than 10 μM (see Table 14). These results show that stable Copper(I) complexes of this invention are potent inhibitors of neutrophil 5-lipoxygenase, thus preventing the accumulation of inflammatory lipid mediators at the sites of inflammation.

Table 14
Inhibition of 5-Lipoxygenase by
Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	30	71.3 ± 2.5
	3.0	29.0 ± 5.0
	0.3	5.5 ± 3.5
	0.03	4.0 ± 1.0
Neocuproine Copper(I) (2:1)	30	99.0 ± 0.6
	3.0	51.0 ± 6.0
	0.3	15.5 ± 2.5
	0.03	7.0 ± 0.0

A similar method was used to determine the inhibition of 5-Lipoxygenase by other stable Copper(I) complexes. The results of these assays are shown summarized below in Table 14a.

Table 14a.
Inhibition of 5-Lipoxygenase
by Stable Copper(I) Complexes

Copper(I) Complex	5-Lipoxygenase Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	9.3
Neocuproine Copper(I) (2:1)	2.7
Biquinoline Copper(I) (2:1)	14
Hexocuproine* Copper(I) (2:1)	5.0
t-Butylcuproine** Copper(I) (1:1)	18

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

Example 10
Inhibition of Leukotriene C_4 Synthetase by Neocuproine and BCDS Copper(I) Complexes (2:1)

Leukotriene C_4 (LTC_4) Synthetase is involved in the formation of LTC_4 from LTA_4, as illustrated in Figure 3, by the addition of a reduced glutathione at the C6 site.

In this example, LTC_4 Synthetase was prepared as a crude fraction from rat basophilic leukemia cells (RBL-1). The crude enzyme fraction was incubated with test compounds, LTA_4 methyl ester, albumin (to stabilize the product), and serine borate (to prevent conversion of LTC_4 to LTD_4) for 15 minutes at 37° C. The reaction was terminated by the addition of ice cold methanol, and LTC_4 concentration was determined by a specific RIA (Bach et al., "Inhibition by Sulfasalazine of LTC4 Synthetase and of Rat Liver Glutathione S-Transferases," Biochem. Pharmacol. 34:2695-2704, 1985; Fitzpatrick et al., "Albumin Stabilizes Leukotriene A4," J. Biol. Chem, 257:4680-4683, 1982).

Both BCDS Copper(I) (2:1) and neocuproine Copper(I) (2:1) were found to be inhibitors of LTC_4 Synthetase with estimated IC50's of 87 and 285 μM, respectively (see Table 15). These results show that stable Copper(I) complexes are potent inhibitors of neutrophil LTC_4 Synthetase, thus preventing the accumulation of inflammatory lipid mediators at the sites of inflammation.

Table 15
Inhibition of Leukotriene C_4 (LTC_4) Synthetase by Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	1000	77.8 ± 1.9
	100	51.0 ± 4.0
	10	26.5 ± 1.5
	1	11.0 ± 2.0
Neocuproine Copper(I) (2:1)	1000	71.0 ± 1.9
	100	32.5 ± 0.5
	10	15.0 ± 1.0
	1	9.0 ± 1.0

Example 11
Inhibition of Elastase by BCDS Copper(I) (2:1)

Proteolysis of various cellular targets by elastase has been implicated in a number of pathologic conditions, including emphysema, rheumatoid arthritis, and psoriasis.

In this experiment, human neutrophil was the source of the elastase. In particular, human neutrophil elastase was prepared in crude form from fresh blood following dextran sedimentation, leukocyte isolation, cell lysis and homogenization of sub-cellular granules containing the elastase. BCDS Copper(I) (2:1) was incubated with the enzyme and substrate (methoxysuccinyl-alanyl-alanyl-propyl-valine-4-nitroanalide) for 8 minutes at 25°C. The reaction is terminated by immersing the test tubes in boiling water for 5 minutes. Spectrophotometric analysis of the proteolytic product is measured at 410 nm (Baugh and Travis, "Human Leukocyte Granule Elastase, Rapid

Isolation and Characterization," Biochemistry 15:836-841, 1976).

BCDS Copper(I) (2:1) was found to inhibit human neutrophil elastase with an estimated IC50 of 12 μM (see Table 16). These results show that stable Copper(I) complexes of this invention are potent inhibitors of neutrophil elastase, thus preventing or limiting the breakdown of normal tissue at the sites of inflammation.

Table 16
Inhibition of Human Neutrophil Elastase by Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	30	65.8 ± 3.1
	3.0	25.0 ± 5.0
	0.3	18.5 ± 0.5
	0.03	5.5 ± 0.5

Example 12
Inhibition of Acetyl Coenzyme A (CoA) Synthetase by Neocuproine and BCDS Copper(I) (2:1)

In this experiment, the ability of two stable Copper(I) complexes, neocuproine Copper(I) (2:1) and BCDS copper (I) (2:1), to inhibit certain key enzymes involved in the formation of lipids is demonstrated.

CoA synthetase (yeast) activity was monitored by utilization of a labeled substrate, sodium [3H]acetate (Grayson and WestKaemper, "Stable Analogs of Acyl Adenylaes, Inhibition of Acetyl and Acyl (acyl-CoA) CoA Synthetase by Adenosine 5'-alkylphosphates," Life Sci. 43: 437-444, 1988). A reaction buffer including 0.1 M glycine-NaOH (pH 9.0), ATP, and the substrate was pre-

incubated for 5 minutes at 27°C, followed by addition of 2 nM coenzyme A for an additional 5 minute incubation at 27° C. The reaction was terminated by addition of HCl, and the remaining substrate determined by scintillation counting.

The results of this experiment are presented in Table 17. Both BCDS Copper(I) (2:1) and neocuproine Copper(I) (2:1) were found to inhibit acetyl CoA synthetase activity.

Table 17
Inhibition of Acetyl CoA Synthetase by
Stable Copper(I) Complexes

Compound	IC_{50} (μM)
BCDS Copper(I) (2:1)	29
Neocuproine Copper(I) (2:1)	47
Reference compounds:	
Ethyl-5-AMP	60
Lovastatin	>100
Orotic Acid	>100

Both stable Copper(I) complexes tested were found to inhibit acetyl CoA synthetase with estimated IC_{50}'s of 30-50 μM. These results indicate that the stable Copper(I) complexes of this invention may serve as lipid modulating (e.g., lipid lowering) agents.

Example 13
Inhibition of HMG-CoA Reductase by Neocuproine and BCDS Copper(I) (2:1)

In this experiment, HMG-CoA reductase was isolated from rat liver and incubated with [^{14}C]HMG-CoA and either neocuproine Copper(I) (2:1) or BCDS Copper(I) (2:1) for 15

minutes at 37°C. The reaction is terminated by addition of HCl, and [^{14}C] MVA is separated from the intact substrate by column filtration (Kubo and Strott, "Differential Activity of 3-hydroxy-3-methylglutaryl Coenzyme A Reductase in Zones of the Adrenal Cortex," Endocrinology 120: 214-221, 1987; Heller and Gould, "Solubilization and Partial Purification of Hepatic 3-hydroxy-3-methylglutaryl Coenzyme A Reductase," Biochem. Biophys. Res. Comm. 50: 859-865, 1973).

Testing at 30 μM indicated that both neocuproine Copper(I) (2:1) and BCDS Copper(I) (2:1) inhibited the HMG-CoA reductase enzyme. The results of this experiment are presented in Table 18.

Table 18
Inhibition of HMG-CoA Reductase by
Stable Copper(I) Complexes

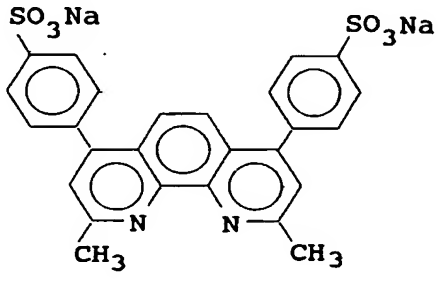
Compound	IC_{50}
BCDS Copper(I) (2:1)	>30 μM, <50 μM
Neocuproine Copper(I) (2:1)	>30 μM, <50 μM
Reference compound:	
Lovastatin	>12 nM

Both stable Copper(I) complexes tested were found to inhibit HMG-CoA reductase with IC_{50}'s estimated at greater than 30 μM. These results indicate that the stable Copper(I) complexes of this invention may serve as lipid modulating (e.g., lipid lowering) agents.

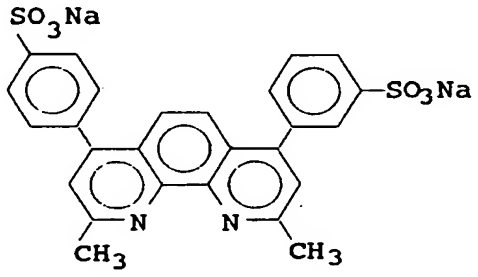
Example 14
Inhibition of HIV-1 Activity by BCDS Copper(I) (2:1) Isomers

The experiments presented in this example demonstrate the effect on anti-HIV activity of different isomers of BCDS Copper(I) (2:1). Two experiments utilized p24 antigen capture as a marker for viral replication, while two further experiments utilized reverse transcriptase activity to monitor the course of infection. The infection in all three experiments was performed in cultures of human peripheral blood mononuclear cells (PBMC) treated with HIV-1.

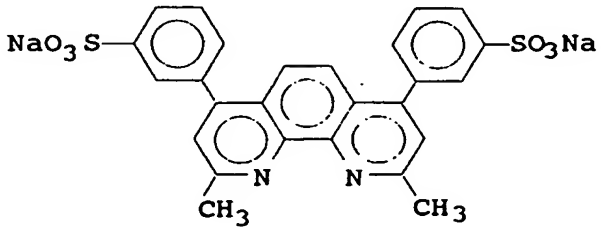
The positional isomers of the BCDS Copper(I) employed in this experiment are identified above as structures IIe, IIe' and IIe'', and are set forth below:



IIe



IIe'



IIe''

Structure IIe is referred to herein as the para-para ("PP") BCDS isomer since both disulfonic acid/sodium salt moieties are located in the para position. Similarly, structure IIe' and IIe'' are referred to herein as the meta-para ("MP") and meta-meta ("MM") BCDS isomers, respectively. In addition, a mixture of the PP, MP and MM BCDS isomers was also tested (referred to herein simply as "BCDS"), having a ratio of PP:MP:MM of approximately 5:39:56.

In the first experiment, the anti-HIV activity of BCDS Copper(I), MP-BCDS Copper(I) and MM-BCDS Copper(I) (2:1) at two concentrations (i.e., 10 and 25 μM) was compared. These concentrations had been previously determined to be partially and completely effective, respectively, for inhibition of HIV replication by BCDS Copper(I).

The same methodology as described above in Example 5 for evaluating inhibition of HIV replication was employed in the experiment. The results of this experiment are present in Table 19.

Table 19

Inhibition of HIV Replication by BCDS Copper(I), MP-BCDS Copper(I) and MM-BCDS Copper(I) (2:1) as Measured by p24 Antigen Capture (@1:1000 viral dilution)

	Week 1		
Compound	p24	(SEM)	% Inhibition
Control (infected cells)	30910.00	3770.00	--
BCDS Copper(I) (10μM)	1959.00	317.16	93.66
BCDS Copper(I) (25μM)	0.25	0.25	99.99
MP-BCDS Copper(I) (10μM)	404.50	124.66	98.69
MP-BCDS Copper(I) (25μM)	0.50	0.50	99.99
MM-BCDS Copper(I) (10μM)	346.50	106.27	98.88
MM-BCDS Copper(I) (25μM)	0.00	0.00	100.00

Compound	Week 2 p24	(SEM)	% Inhibition
Control (infected cells)	10483.80	1109.73	--
BCDS Copper(I) (10μM)	3286.00	242.36	68.66
BCDS Copper(I) (25μM)	0.00	0.00	100.00
MP-BCDS Copper(I) (10μM)	901.75	277.26	91.40
MP-BCDS Copper(I) (25μM)	0.00	0.00	100.00
MM-BCDS Copper(I) (10μM)	549.50	176.25	94.76
MM-BCDS Copper(I) (25μM)	0.00	0.00	100.00

In a second experiment, the activity of BCDS Copper(I) and PP-BCDS Copper(I) was compared in the manner described above. The results of this experiment are set forth in Table 20. In this experiment the p24 concentrations were lower than in the above experiment. This is due to a different ELISA technique used in this experiment. The standard curve for p24 detection maximizes at 300 pg/ml. Any values over 300 require a kinetic extrapolation to estimate the p24 concentration. Such extrapolation gives a substantial underestimation of the actual p24 concentration. To obtain a more accurate estimate, a series of dilutions of the sample was made to arrive at a reading that is in the middle of the standard curve, and the dilution factor applied to the reading to give the p24 concentrations. This method (which was used in the first experiment, see Table 19 above) while more accurate, yields an overestimate due to the errors of dilution. Nevertheless, the comparisons from one sample to the next in each experiment reflect the inhibitory effects of stable Copper(I) complexes tested.

Table 20
Inhibition of HIV Replication by BCDS Copper(I) and PP-BCDS Copper(I) (2:1) as Measured by p24 Antigen Capture (@1:1000 viral dilution)

	Week 1		
Compound	p24	(SEM)	% Inhibition
Control (infected cells)	1649.75	29.32	--
BCDS Copper(I) (10μM)	474.25	41.22	71.25
BCDS Copper(I) (25μM)	39.50	6.06	97.61
PP-BCDS Copper(I) (10μM)	480.00	49.65	70.90
PP-BCDS Copper(I) (25μM)	34.50	4.57	97.91

	Week 2		
Compound	p24	(SEM)	% Inhibition
Control (infected cells)	2256.50	45.93	--
BCDS Copper(I) (10μM)	1785.00	49.03	20.90
BCDS Copper(I) (25μM)	22.00	6.38	99.02
PP-BCDS Copper(I) (10μM)	1915.75	69.75	15.10
PM-BCDS Copper(I) (25μM)	33.25	6.60	98.53

In a third experiment, the anti-HIV activity of BCDS Copper(I), PP-BCDS Copper(I), MP-BCDS Copper(I) and MM-BCDS Copper(I) (2:1) was determined by monitoring the same type of culture (i.e., HIV-1, PBMC) by measuring the reverse transcriptase activity as an infection marker. The PBMC culture conditions for this experiment are described above in Example 5. Following 6 days of incubation, the activity of HIV-1 reverse transcriptase in cellular extracts was determined as a marker for the replication of the virus in culture. The measurement of HIV-1 reverse transcriptase in PBMC cultures may be performed by known techniques (Chattopadhyay et al., "Purification and Characterization of Heterodimeric Human Immunodeficiency Virus Type 1 (HIV-1) Reverse Transcriptase Produced by an In Vitro Processing of p66

with Recombinant HIV-1 Protease," J. Biol. Chem. 267:14227-14232, 1992). The results of this experiment are presented in Table 21.

Table 21

Inhibition of HIV Replication by BCDS Copper(I), PP-BCDS Copper(I), MP-BCDS Copper(I) and MM-BCDS Copper(I) (2:1) as Measured by Reverse Transcriptase Activity

		Reverse Transcriptase Activity	
Compound	Conc. (μM)	CPM	% Inhibition
None(control)	0	29283	NA
BCDS Copper(I)	0.001	23963	18.17
	0.01	19585	33.12
	0.1	17340	40.78
	1	17623	39.82
	10	4974	83.01
	100	585	98.00
PP-BCDS Copper(I)	0.001	26934	8.02
	0.01	28097	4.05
	0.1	12742	56.49
	1	12247	58.18
	10	1846	93.70
	100	566	98.07
MP-BCDS Copper(I)	0.001	19966	31.82
	0.01	15040	48.64
	0.1	12369	57.76
	1	9880	66.26
	10	1408	95.19
	100	540	98.16
MM-BCDS Copper(I)	0.001	22679	22.55
	0.01	18212	37.81
	0.1	18464	36.95
	1	2085	92.88
	10	583	98.01

In a fourth experiment, inhibition of HIV-1, HIV-2 and SIV, as compared to AZT, was determined for BCDS Copper(I), PP-BCDS Copper(I), MP-BCDS Copper(I) and MM-BCDS Copper(I). The experimental conditions described above were employed utilizing a Reverse Transcription assay to monitor infection. The results of this experiment are presented in Table 22. It should be noted that the data presented in Table 22 are reported in a different format from that of Table 21. In particular, the data of Table 22 represent the calculated EC_{50} values. The EC_{50} is determined by non-linear regression from inhibition data (such as that presented in Table 21), and extrapolated for the concentration of the test compound required to accomplish a 50% inhibition of reverse transcriptase activity.

Table 22
Inhibition of HIV-1, HIV-2 and SIV Replication by BCDS Copper(I), PP-BCDS Copper(I), MP-BCDS Copper(I) and MM-BCDS Copper(I) (2:1) as Measured by Reverse Transcriptase Activity

	EC50 (μM)		
Compound	HIV-1	HIV-2	SIV
BCDS Copper(I)	1.7	17.6	4.6
PP-BCDS Copper(I)	0.25	1.2	6.4
MP-BCDS Copper(I)	0.04	12.1	4.3
MM-BCDS Copper(I)	0.13	0.62	6.1
AZT	0.004-0.009	0.0004	0.0066

Example 15
Anti-Viral Activity of Stable Copper(I) Complexes

This example illustrates that the stable copper (I) compounds of this invention, as well as the free ligands, have general anti-viral activity. In this experiment, BCDS Copper(I) and BCDS alone (i.e., the free ligand) were assayed for the ability to inhibit the murine virus encephalomyocarditis (EMCV) and the cytomegalo virus (CMV).

Inhibition of EMCV

Cultures of A_{549} cells (human lung) were infected with EMCV for 24-48 hours in the presence of either BCDS Copper(I) or BCDS alone. The cells were cultured in DMEM (10% FBS) for 3-4 days prior to use. The medium was then removed, and the cells incubated with sufficient EMCV in serum free DMEM to kill between 30-90% of the cells in the culture. After 2-3 hours of incubation of the cells with EMCV in their presence (or absence) of the test compounds, complete medium (DMEM + 10% FBS) was added and the cells allowed to incubate for 1-2 days in the presence or absence of the test compounds at concentrations ranging from 0.0001-0.0005 M.

The viability of the cultures was then measured by mitochondrial function test (Mossman, J. Immunol. Meth. 65:55-63,1983). The ability of the test compounds to protect the cells from the lethality of the EMCV infection was calculated as a percent protection compared to the mitochondrial activity of parallel, uninfected cells. The results of this experiment are presented in Table 23.

Table 23
Inhibition of EMCV by
Stable Copper(I) Complex and Free Ligand

	% Protection	
Conc. (μM)	BCDS Copper(I) (2:1)	BCDS Ligand
100	30.2	10.7
200	67.2	3.2
400	97.7	22.2
500	153.2	84.2

Inhibition of CMV

Normal Diploid Human Fibroblasts were isolated and cultured with Minimal Essential Medium (MEM) containing Earles balanced salts and supplemented with 10% Fetal Bovine Serum (FBS). Cytomegalo virus (CMV) was added to the cultures in the presence or absence of BCDS and BCDS Copper(I) (2:1). Five cultures were employed in each test group, with the exception of the uninfected cell groups which utilized 8 cultures. The uninfected cell groups were used to ensure that antiviral activity was achieved in the absence of any direct cytotoxic effect of the test compounds.

After one week of incubation, cellular viability (i.e., mitochondrial function) was determined, and the ability of the test compounds to prevent the cytopathic effect (CPE) of the virus was calculated as percent protection by the following formula:

$$\% \text{ Protection} = (V_t - V_v)/V_u - V_v) \times 100$$

where V_t represents viability of the test culture, V_v represents the viability of culture with virus alone, and V_u represents the viability of uninfected cells.

The results of this experiment are presented in Table 24. No cytotoxic effects were observed on the uninfected compounds treated with the test compounds.

Table 24
Inhibition of CMV by
Stable Copper(I) Complex and Free Ligand

Conc. (μM)	% Protection (SEM) BCDS Copper(I) (2:1)	BCDS Ligand
25	13.1 (7.7)	34.2 (8.1)
100	117.3 (13.3)	35.4 (6.2)
250	92.9 (5.2)	23.6 (8.3)

Example 16
Inhibition of HIV-1 and HIV-2 Proteases by Stable Copper(I) Complexes

This example illustrates the ability of stable Copper(I) complexes of this invention to inhibit HIV-1 and HIV-2 proteases.

HIV-1 Protease ^{125}I-SPA Assay

In this experiment, SPA beads (Scintillation Proximity Assay) were coupled with a peptide substrate to assay for HIV-1 protease. The substrate was a 12 residue peptide with the following sequence:

AcN-Tyr-Arg-Ala-Arg-Val-Phe-Phe-Val-Arg-Ala-Ala-Lys-COOH

The peptide was monoiodinated on the terminal tyrosine residue, biotinylated through the ε-amino group on the terminal lysine, and linked to the SPA bead via a streptavidin link.

HIV-1 protease cleaves the peptide substrate at the Phe-Phe bond, releasing the ^{125}I-fragment from the bead. Once the peptide is cleaved, it can no longer stimulate the scintillant in the SPA bead and the signal is reduced.

The rate of reduction is proportional to the activity of the HIV-1 protease. Recombinant HIV-1 protease, affinity purified for kinetic and assay studies, was used in this experiment.

Two types of controls were conducted with this assay, one without enzyme to test for possible scintillation quenching by the test compound (i.e., BCDS Copper(I) (2:1)), and another positive control with acetyl pepstatin. At concentrations 10 times that used in the assay, there was no quench detected in the presence of BCDS Copper(I) (2:1).

The results of this experiment are presented in Table 25. The data presented is the mean ± SD of the percent inhibition relative to a no enzyme control reaction. As discussed above, the IC_{50} was estimated from the point at which the dose inhibition line crossed the 50% inhibition line. The estimated IC_{50} with this HIV-1 protease assay was 11μM.

Table 25
Inhibition of HIV-1 Protease by
a Stable Copper(I) Complex

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	25	86.7 ± 2.1
	10	45.2 ± 2.3
	5	17.6 ± 2.3
	2	12.2 ± 1.8
	1	8.5 ± 4.6
	0.5	1.8 ± 0.6
	0.1	0.0 ± 1.9
Reference Compound:		
Acetyl Pepstatin	0.5	67.4 ± 1.1
	0.25	50.1 ± 0.4
	0.1	28.4 ± 7.7

0.05	16.6 ± 0.5
0.025	10.2 ± 2.6
0.01	2.4 ± 3.5

HIV-2 Protease ^{125}I-SPA Assay

As in the above experiment, SPA beads were coupled with a peptide substrate to assay for HIV-2 protease. The substrate was the 12 residue peptide identified above and monoiodinated on the terminal tyrosine residue, biotinylated through the ε-amino group on the terminal lysine, and linked to the SPA bead via a streptavidin link.

HIV-2 protease cleaves the peptide substrate at the Phe-Phe bond, releasing the ^{125}I-fragment from the bead. Once the peptide is cleaved, it can no longer stimulate the scintillant in the SPA bead and the signal is reduced. The rate of reduction is proportional to the activity of the HIV-2 protease. Recombinant HIV-2 protease, affinity purified for kinetic and assay studies, was used in this experiment. HIV-2 protease has about 50% sequence homology with HIV-1 protease, and is similar to simian immunodeficiency virus (SIV) protease.

Two types of control assays were again run, one without enzyme and the other using acetyl pepstatin as a positive control.

The results of this experiment are presented in Table 26. The data presented is the mean ± SD of the percent inhibition relative to a no enzyme control reaction. The IC_{50} was estimated from the point at which the dose inhibition line crossed the 50% inhibition line. The estimated IC_{50} with this HIV-2 protease assay was 10μM.

Table 26
Inhibition of HIV-2 Protease by
a Stable Copper(I) Complex

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	25	51.9 ± 5.5
	10	49.6 ± 2.9
	5	32.2 ± 2.8
	2	14.1 ± 1.1
	1	5.3 ± 0.9
	0.5	0.8 ± 5.3
	0.1	2.5 ± 0.6
Reference Compound:		
Acetyl Pepstatin	5.0	88.8 ± 0.5
	2.5	70.6 ± 2.3
	1.0	45.5 ± 1.8
	0.5	37.2 ± 0.3
	0.25	19.9 ± 5.6
	0.1	4.3 ± 12.3

Example 17
Inhibition of HIV Reverse Transcriptase by Stable Copper(I) Complexes

This example illustrates the ability of a stable Copper(I) complex of this invention, BCDS Copper(I) (2:1), to inhibit HIV reverse transcriptase activity.

As in Example 16 above, SPA (Scintillation Proximity Assay) beads were used to assay for the reverse transcriptase activity. The reverse transcriptase (10 uL) was incubated with the 3H-deoxyribonucleotides (10 uL), the DNA primer linked to biotin (10 uL), and the RNA template. After incubation at 37° for 20 minutes, the reaction was stopped and the labeled product was recovered

by addition of the SPA beads coupled to streptavidin which binds to the biotin linked DNA primer.

The extent of the reaction was determined by scintillation counting. Increasing concentrations of BCDS Copper(I) (2:1) were added and the extent of the reaction determined by the method described above.

The results of this experiment are presented in Table 27. The data show the mean ± SD of the percent inhibition relative to a no test compound control reaction. The IC_{50} is estimated from the point at which the dose inhibition like crosses the 50% inhibition line. The estimated IC_{50} was 11μM.

Table 27
Inhibition of HIV Reverse Transcriptase by a Stable Copper(I) Complex

Compound	Conc. (μM)	Percent Inhibition (Mean)
BCDS Copper(I) (2:1)	25	63.2
	10	35.4
	5	26.8
	2	27.0
	1	9.8
	0.5	0.2
	0.1	1.8

Example 18
Inhibition of Protein Kinase C by Stable Copper(I) Complexes

This example illustrates the ability of representative stable Copper(I) complexes to inhibit enzymes involved in intracellular signal transduction.

The enzymes tested in this experiment were various protein kinase C isozymes.

Protein Kinase C (non-selective) Assay

In this experiment, the reaction mixture included 20 mM Tris-HCl, pH 7.4, [32P]-ATP, phosphatidylserine, partially purified PKC from rat brain, and one of the test compounds (Hunnun et al., "Activation of Protein Kinase C by Triton X-100 Mixed Micelles Containing Diacylglycerol and Phosphatidylserine," J. Biol. Chem. 260:10039-10043, 1985; Jeng et al., "Purification of Stable Protein Kinase C from Mouse Brain Cytosol by Specific Ligand Elution Using Fast Protein Liquid Chromatography," Cancer. Res. 46:1966-1971, 1986). Following a 10 minute incubation, 25 ul aliquots are removed, spotted on phosphocellulose paper, washed three times in cold phosphoric acid, dried, and counted to determine phosphorylated product. The results of this experiment are presented in Table 28.

Table 28
Inhibition of Protein Kinase C (non-selective) by Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	300	87.5 ± 2.7
	30	9.5 ± 4.5
	3.0	7.5 ± 4.5
	0.3	2.5 ± 4.5
Neocuproine Copper(I) (2:1)	300	62.0 ± 1.9
	30	22.0 ± 7.0
	3.0	6.0 ± 4.0
	0.3	-12.0 ± 2.0

A similar method was used to determine the inhibition of Protein Kinase C (non-selective) by other stable Copper(I) complexes. The results of these assays are shown below in summary Table 28a.

Table 28a.
Inhibition of Protein Kinase C (non-selective)
by Stable Copper(I) Complexes

Compound	Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	97
Neocuproine Copper(I) (2:1)	145
Biquinoline Copper(I) (2:1)	>>300
Hexocuproine* Copper(I) (2:1)	>>300
t-Butylcuproine** Copper(I) (1:1)	92

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

Protein Kinase Cα Assay

Protein Kinase Cα is one of the major protein kinase C isoforms. Protein kinase C is a family of serine/threonine protein kinases that mediate the actions of a wide variety of growth factor, hormone, and neurotransmitter action.

In this experiment, protein Kinase Cα was purified to homogeneity from rat brain using a modification of a the published procedure(3). The purity of the isolated PKCα was confirmed by SDS/polyacrylamide gel electrophoresis and isoform-specific antibodies. The enzyme was pre-incubated with the test compounds, and its activity is measured by the ability of the enzyme to phosphorylate histone H1 in the absence and presence of calcium, phosphatidylserine, diolein and [32P]ATP. Following a 5

minute incubation, the reaction was terminated by the addition of acetic acid, 50 ul aliquots are removed, spotted on phosphocellulose paper, washed three times in water, dried, and counted to determine phosphorylated product. The data presented in Table 29 show that the addition of the stable Copper(I) complexes inhibit the activity of Protein Kinase Cα.

Table 29
Inhibition of Protein Kinase Cα by
Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	100	88.3 ± 0.6
	10	18.0 ± 2.0
	1.0	0.0 ± 3.0
	0.1	-4.5 ± 6.5
Neocuproine Copper(I) (2:1)	100	87.5 ± 1.8
	10	23.6 ± 4.5
	1.0	-1.5 ± 3.5
	0.1	-5.0 ± 1.0

A similar method was used to determine the inhibition of Protein Kinase Cα by other stable Copper(I) complexes. The results of these assays are shown below in summary Table 29a.

Table 29a.
Inhibition of Protein Kinase Cα
by Stable Copper(I) Complexes

Compound	Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	28
Neocuproine Copper(I) (2:1)	25
Biquinoline Copper(I) (2:1)	14
Hexocuproine* Copper(I) (2:1)	5.5
t-Butylcuproine** Copper(I) (1:1)	17

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

Protein Kinase Cβ Assay

Protein Kinase Cβ is another major protein kinase C isoforms. Protein kinase C is a family of serine/threonine protein kinases that mediate the actions of a wide variety of growth factor, hormone, and neurotransmitter action.

In this experiment, Protein Kinase Cβ (which includes βI and βII forms) was purified to homogeneity from rat brain using a modification of a published protocol (Woodgett and Hunter, "Isolation and Characterization of Two Distinct Forms of Protein Kinase C," J. Biol. Chem. 262:4836-4848, 1987). The purity of the isolated PKCα was confirmed by SDS/polyacrylamide gel electrophoresis and isoform-specific antibodies. The enzyme was pre-incubated with test compounds, and its activity is measured by the ability of the enzyme to phosphorylate histone H1 in the absence and presence of calcium, phosphatidylserine, diolein and [32P]ATP. Following a 5 minute incubation, the reaction was terminated by the addition of acetic acid, 50 ul aliquots are removed, spotted on

phosphocellulose paper, washed three times in water, dried, and counted to determine phosphorylated product.

The data presented in Table 30 show that the addition of the stable Copper(I) complexes inhibit the activity of Protein Kinase Cβ.

Table 30
Inhibition of Protein Kinase Cβ by
Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	100	96.8 ± 2.0
	10	20.0 ± 2.0
	1.0	3.5 ± 6.5
	0.1	6.5 ± 4.5
Neocuproine Copper(I) (2:1)	100	84.5 ± 1.9
	10	25.5 ± 1.5
	1.0	4.0 ± 7.0
	0.1	3.5 ± 6.5

Protein Kinase Cγ Assay

Protein Kinase Cγ is another major protein kinase C isoform. Protein kinase C is a family of serine/threonine protein kinases that mediate the actions of a wide variety of growth factor, hormone, and neurotransmitter action.

In this experiment, Protein Kinase Cγ was purified from insect cells expressing a baculovirus recombinant rabbit brain protein kinase Cγ isoform. The enzyme was pre-incubated with the test compounds, and its activity was measured by the ability of the enzyme to phosphorylate histone H1 in the absence and presence of calcium, phosphatidylserine, diolein and [32P]ATP. Following a 5 minute incubation, the reaction was terminated by the addition of acetic acid, 50 ul aliquots were removed, spotted on phosphocellulose paper, washed three times in

water, dried, and counted to determine phosphorylated product.

The data presented in Table 31 show that the addition of the stable Copper(I) complexes inhibit the activity of Protein Kinase Cγ.

Table 31
Inhibition of Protein Kinase Cγ by
Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean)
BCDS Copper(I) (2:1)	100	99
	10	51
	1.0	21
	0.1	5
Neocuproine Copper(I) (2:1)	100	97
	10	40
	1.0	28
	0.1	17

The data in Tables 28-31 show that the stable Copper(I) complexes of this invention are potent inhibitors of Protein Kinase C.

Example 19
Inhibition of Protein Tyrosine Kinases by
Stable Copper(I) Complexes

This example illustrates the ability of representative stable Copper(I) complexes to inhibit enzymes involved in intracellular signal transduction. The enzymes tested in this experiment were protein tyrosine kinases specific for growth factors and cytokines.

Epidermal Growth Factor (EGF)
Receptor Tyrosine Kinase (human recombinant) Assay

The binding of EGF or TGF-α (Transforming Growth Factor α) to the EGF receptor results in activation of the tyrosine kinase portion of the receptor. This kinase phosphorylates several cytosolic proteins which lead to induction of intracellular signaling pathways eventually leading to cell mitogenesis and in some cases cellular transformation. Inhibition of the EGF tyrosine kinase is useful for chemotherapy for malignant cells.

In this experiment, a recombinant form of the human Epidermal Growth Factor Tyrosine Kinase domain was assayed (Geissler et al., "Thiazolidine-Diones:Biochemical and Biological Activity of a Novel Class of Tyrosine Protein Kinase Inhibitors," J. Biol. Chem. 165:22255-22261, 1990; Wedegartner and Gill, "Activation of the Purified Protein Kinase Domain of the Epidermal Growth Factor Receptor," J. Biol. Chem. 264:11346-11353, 1989; Yaish et al., "Blocking of EGF-dependent Cell Proliferation by EGF-Receptor Kinase Inhibitors," Science 242:933-935, 1988).

The kinase assay measures the activity of the 69kD kinase domain by employing an immobilized synthetic polypeptide as a substrate. Following a 10 minute reaction, phosphorylated tyrosine residues were detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody was quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin linked β-galactosidase enzyme. Fluorescence resulting from conversion of fluoroscein-di-β-galactoside to fluorescein was measured. The results of this experiment are presented in Table 32.

Table 32
Inhibition of Epidermal Growth Factor (EGF) Receptor Tyrosine Kinase (human recombinant) by Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	10	102.3 ± 2.3
	1	40.0 ± 3.6
	0.1	11.7 ± 2.7
	0.01	0.3 ± 2.4
Neocuproine Copper(I) (2:1)	10	96.7 ± 1.0
	1	43.7 ± 5.5
	0.1	12.0 ± 4.0
	0.01	-5.7 ± 0.9

A similar method was used to determine the inhibition of Epidermal Growth Factor (EGF) Receptor Tyrosine Kinase (human recombinant) by other stable Copper(I) complexes. The results of these assays are shown below in summary Table 33.

Table 33
Inhibition of Epidermal Growth Factor (EGF) Receptor Tyrosine Kinase (human recombinant) by Stable Copper(I) Complexes

Compound	Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	1.3
Neocuproine Copper(I) (2:1)	1.4
Biquinoline Copper(I) (2:1)	2.4
Hexocuproine* Copper(I) (2:1)	2.3
t-Butylcuproine** Copper(I) (1:1)	3.0

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

p56lck Tyrosine Kinase Assay

The lck tyrosine kinase is a member of the src family of cytoplasmic tyrosine kinases. It is expressed only in T-lymphocytes and NK cells. The p56lck Tyrosine Kinase is a 56 kD protein that is found associated with the cytoplasmic side of the plasma membrane of these cells. It is responsible of transmission of the IL-2 signal leading to T-lymphocyte activation. The binding of IL-2 to specific IL-2 receptors leads to activation of the p56 tyrosine kinase. In addition, the p56lck Tyrosine Kinase has been found to function in signal transduction for antigen activated CD4 and CD8 T-cell receptors.

In this experiment, the p56lck Tyrosine Kinase was purified from bovine thymus. The kinase assay measures the activity of the 69kD kinase domain by employing an immobilized synthetic polypeptide as a substrate. The test compounds were pre-incubated with the enzyme for 15 minutes. Following a 10 minute reaction with 100 μM ATP, phosphorylated tyrosine residues are detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody was quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin linked β-galactosidase enzyme. Fluorescence resulting from conversion of fluoroscein-di-β-galactoside to fluorescein was measured (Hatekeyama et al., "Interaction of the IL-2 Receptor with the src-Family Kinase p56lck: Identification of Novel Intermolecular Association," Science 252:1523-1528, 1991; Caron et al., "Structural Requirements for Enhancement of T-cell Responsiveness by the Lymphocyte Specific Tyrosine Protein Kinase p56lck," Mol. Cell Biol. 12:2720-2729, 1992; Cheng et al., "A Synthetic Peptide Derived from p34cdc2 is a Specific and Efficient Substrate of src-

Family Tyrosine Kinases." J. Biol. Chem. 267:9248-9256, 1992).

Both the BCDS Copper(I) and neocuproine Copper(I) complexes were found to be potent inhibitors of the kinase activity. The results of this experiment are presented in Table 34.

Table 34
Inhibition of $p56^{lck}$ Tyrosine Kinase Activity by Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	10	97.5 ± 1.7
	1	19.5 ± 1.5
	0.1	-3.5 ± 0.5
	0.01	2.5 ± 7.5
Neocuproine Copper(I) (2:1)	10	90.0 ± 2.3
	1	19.5 ± 0.5
	0.1	-8.0 ± 2.0
	0.01	-1.0 ± 6.0

A similar method was used to determine the inhibition of $p56^{lck}$ Tyrosine Kinase by other stable Copper(I) complexes. The results of these assays are shown below in summary Table 34a.

Table 34a.
Inhibition of $p56^{lck}$ Tyrosine Kinase by Stable Copper(I) Complexes

Compound	Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	2.4
Neocuproine Copper(I) (2:1)	2.7
Biquinoline Copper(I) (2:1)	2.6

Hexocuproine* Copper(I)	(2:1)	0.3
t-Butylcuproine** Copper(I)	(1:1)	1.9

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

p59^{fyn} Tyrosine Kinase Assay

The fyn tyrosine kinase is also a member of the src family of non-receptor linked cytoplasmic tyrosine kinases. The p59^{fyn} Tyrosine Kinase is responsible for mediating signal transduction through the T-cell receptor (TCR). This receptor is responsible for a signal cascade leading to lymphokine secretion and cell proliferation. The p59^{fyn} Tyrosine Kinase is also one of several kinases associated with the B-cell receptor.

In this experiment, the p59^{fyn} Tyrosine Kinase was purified from bovine thymus. The kinase assay measures the activity of the 69kD kinase domain by employing an immobilized synthetic polypeptide as a substrate. The test compounds are preincubated with the enzyme for 15 minutes. Following a 10 minute reaction with 100 μM ATP, phosphorylated tyrosine residues are detected by incubation with a monoclonal anti-phosphotyrosine antibody. Bound anti-phosphotyrosine antibody is quantitated by incubation with a biotin-linked anti-mouse IgG, followed by streptavidin linked β-galactosidase enzyme. Fluorescence resulting from conversion of fluoroscein-di-β-galactoside to fluorescein is measured (Cooke et al., "Regulation of T-cell Receptor Signaling by a src Family Protein Tyrosine Kinase p59^{fyn}," Cell 65:281-291, 1991; Grassman et al., "Protein Tyrosine Kinase p59^{fyn} is Associated with the T-cell Receptor CD3 Complex in Functional Human Lymphocytes," Eur. J. Immunol. 22:283-286, 1992; Appleby et al., "Defective T-cell Receptor Signaling in Mice Lacking the Thymic Isoform of p59^{fyn}," Cell 70:751-763, 1992). Both the BCDS Copper(I) and neocuproine Copper(I) complexes were found to be

potent inhibitors of the kinase activity. The results of this experiment are presented in Table 35.

Table 35
Inhibition of $p59^{fyn}$ Tyrosine Kinase Activity by Stable Copper(I) Complexes

Compound	Conc. (μM)	Percent Inhibition (Mean ± SEM)
BCDS Copper(I) (2:1)	10	99.0 ± 2.7
	1	38.0 ± 5.0
	0.1	20.5 ± 0.5
	0.01	2.0 ± 8.0
Neocuproine Copper(I) (2:1)	10	91.0 ± 3.0
	1	25.5 ± 1.5
	0.1	1.0 ± 6.0
	0.01	2.5 ± 4.5

A similar method was used to determine the inhibition of $p59^{fyn}$ Tyrosine Kinase by other stable Copper(I) complexes. The results of these assays are shown below in summary Table 35a.

Table 35a.
Inhibition of $p59^{fyn}$ Tyrosine Kinase by Stable Copper(I) Complexes

Compound	Inhibition IC_{50} (μM)
BCDS Copper(I) (2:1)	1.5
Neocuproine Copper(I) (2:1)	2.4
Biquinoline Copper(I) (2:1)	0.2
Hexocuproine Copper(I) (2:1)	0.4

t-Butylcuproine** Copper(I)	(1:1)	0.2

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

The data in Tables 33-35 were used to determine the 50% inhibitory dose (IC_{50}) of each stable Copper(I) complex with each protein tyrosine kinase tested. This data is shown in Table 36. These results show that the stable Copper(I) complexes of this invention are potent inhibitors of this class of tyrosine kinase.

Table 36
Inhibition of Protein Tyrosine Kinases by Stable Copper(I) Complexes

Compound		Inhibition (IC_{50} μM)		
		EGF-Receptor	$p56^{lck}$	$p59^{fyn}$
BCDS Copper(I)	(2:1)	1.3	2.4	1.5
Neocuproine Copper(I)	(2:1)	1.4	2.7	2.4
Biquinoline Copper(I)	(2:1)	2.4	2.6	0.2
Hexocuproine* Copper(I)	(2:1)	2.3	0.3	0.4
t-Butylcuproine** Copper(I)	(1:1)	3.0	1.9	0.2

*2,9-dihexyl-1,10-phenanthroline
**2,9-di-t-butyl-1,10-phenanthroline

Example 20
Inhibition of Syncytium Formation by Stable Copper(I) Complexes

This example illustrates the effect of a representative stable Copper(I) complex of this invention (BCDS Copper(I)) on syncytium formation using a virus-free, genetically engineered syncytium formation assay which relies only upon the molecular recognition of gp120,

gp41 and the CD4 receptor to create the syncytium (Fu et al., J. Virol 7:3818, 1993). The CEM cell (a T lymphoblastoid human leukemia cell line), which carries the CD4 receptor and is also infectable by HIV-1, was incubated with a genetically engineered cell-line (TF228.1.16). The TF cell expresses gp160, which is processed by the cellular proteases into gp120 and gp41 and inserted onto the surface of the cell. Within a short time, syncytium will form and can be quantified microscopically.

The data from a series of BCDS Copper(I) complexes which differ only in the positions of the sulfonate moieties on the phenyl group of BCDS (i.e., structures IIe, IIe' and IIe" above), as well as a mixture of the above complexes, are presented in Table 37. This data demonstrates the ability of the stable Copper(I) complexes to inhibit syncytium formation relative to two positive controls (i.e., dextran sulfate and HPA-23) and free BCDS.

Table 37
TF-CEM Syncytium Formation Assay

Compound	Syncytium Formation (IC_{50} μM)
BCDS Copper(I)	1.97 (n=5)
PP-BCDS Copper(I)	2.40 (n=2)
MP-BCDS Copper(I)	1.28 (n=2)
MM-BCDS Copper(I)	1.70 (n=2)
BCDS (no copper)	>100 (n=3)
Dextran Sulfate	0.28 (n=2)
HPA-23	1.01 (n=4)

Thus, it is believed that BCDS Copper(I) inhibits HIV-replication via prevention of viral entry, possibly by interacting with the viral proteins gp120 and gp41, and preventing their functions in viral binding and membrane fusion. These data have strong implication for the

utility of BCDS Copper(I) in preventing the spread of HIV to uninfected cells.

Example 21
Viral Inhibition by Stable Copper(I) Complexes

This example illustrates the ability of representative stable Copper(I) complexes of this invention to inhibit various immunodeficiency viruses: HIV-1 ($HIV\text{-}1_{LAV}$); HIV-2 ($HIV\text{-}2_{ROD2}$); an FTC resistant strain of HIV-1 which is not resistant to AZT ($HIV\text{-}1_{FTC^R}$); and SIV (SIV_{SMN}). The PBMC culture conditions for this experiment are described above in Example 5. Following 6 days of incubation, the activity of HIV-1 reverse transcriptase in cellular extracts was determined as a marker for the replication of the virus in culture. The measurement of HIV-1 reverse transcriptase in PBMC cultures may be performed by known techniques (Chattopadhyay et al., J. Biol. Chem. 267:14227-14232, 1992). Human Peripheral Blood Monocytes (PBM) are isolated by standard gradient techniques from whole blood. The PBM cultures are stimulated with interleukin 2 and treated with an innoculum of the various strains of immunodeficiency viruses. After a period of 5-6 days, the cells extracts are prepared and analyzed for reverse transcriptase activity via incorporation of thymidine into DNA. The results of this experiment are presented in Table 38.

Table 38
Inhibition of Immunodeficiency Virus by Stable Copper(I) Complexes

	EC_{50} Values (μM)			
Compound	$HIV\text{-}1_{LAV}$	$HIV\text{-}2_{ROD2}$	$HIV\text{-}1_{FTC^R}$	SIV_{SMN}
BCDS Copper(I)	1.7	17.6	4.6	16.1
PP-BCDS Copper(I)	0.25	1.2	6.4	5.75

MP-BCDS Copper(I)	0.04	12.1	4.3	8.48
MM-BCDS Copper(I)	0.13	0.62	6.1	10.1

These data demonstrate that BCDS Copper(I) complexes inhibit several immunodeficiency viruses including HIV-1, HIV-2, and SIV. HIV-1_{FTC}^{R} is a strain of HIV-1 which was selected to be resistant to a drug called FTC. The inhibition of the FTC resistant strain by BCDS Copper(I) indicates that BCDS Copper(I) does not share the same reistance functions against HIV-1 that FTC does.

In the following experiment, the stable Copper(I) complexes of this invention were also active against two low passage clinical isolates of HIV-1 as shown by the results presented in Table 39. In this experiment, the same techniques were used as in Example 14, Table 19 and p24 antigen capture was used to quantitate the amounts of HIV in the PBMC culture. The viral strains (# 301660 and # 301723) are derived from early passage (less than 2 months) clinical isolates, selected for aggressive growth in PBMC from the NIH AIDS Reference Reagent Program. The stable Copper(I) complex tested in this experiment was BCDS Copper(I).

Table 39
Inhibition of Clinical Isolates of HIV-1 by
a Stable Copper(I) Complex

Strain # 301660		Strain # 310723	
Concentration (μM)	% Control	Concentration (μM)	% Control
5	51.2	5	52.6
10	44.0	10	3.5
25	22.6	25	.07
50	1.5	50	.04
75	0.02	75	.05

Furthermore, stable Copper(I) complexes can inhibit macrophage tropic strains, as shown by the data presented in Table 40.

Table 40
Inhibition of HIV-1 in
CSF Stimulated Human Macrophages by
Stable Copper(I) Complexes

Compound	EC_{50}
BCDS Copper(I)*	4.5
PP-BCDS Copper(I)	1.7
MP-BCDS Copper(I)	1.6
MM-BCDS Copper(I)	>100**

* Also active against HIV-1Bal in Human Macrophages
** 5% inhibition at 10μM

In this experiment, parallel cultures of uninfected cells were used to assess direct cytotoxic effects, and to demonstrate selective inhibition of HIV. For example, the results of an experiment directed to PBMC proliferation are presented in Table 41. Examination of DNA synthesis and/or mitochondrial function confirm that BCDS Copper(I) inhibits HIV without harming the infected cell. Isolation and culture of PBMC is as described in Example 5. In this experiment, the cells are counted at the start, and all cultures (treated or untreated) begin with the same cell number. The cells are counted again after 5 days. The results are expressed as: % Control = (cells/ml (treated culture)/cells/ml (untreated culture))*100. These data show that there is no effect on PBMC proliferation in the presence of as much as 100 μM BCDS Copper(I) complexes over the 5 day period, which indicates that BCDS Copper(I) complexes selectively inhibit the replication of HIV and other viruses in PBMC with no accompanying interference with cellular proliferation.

Table 41
Effect of Stable Copper(I) Complexes on PBMC Proliferation

Concentration (μM)	BCDS Copper(I) Mixture*	BCDS Copper(I) Structure IIe	BCDS Copper(I) Structure IIe'	BCDS Copper(I) Structure IIe"
1	100	100	100	100
10	100	100	100	100
100	98.7	114.9	88.1	100.7

Example 22
Inhibition of Pathological Human Viruses by Stable Copper(I) Complexes

This example illustrates the inhibitory activity of stable Copper(I) complexes of this invention on Respiratory Syncytium Virus (RSV), Hepatitis B Virus (HBV), Influenza A Virus (IF-A) and Influenza B Virus (IF-B). The results of these experiments are presented in Table 42.

Table 42
Inhibition of RSV, HBV, IF-A and IF-B

Compound	Virus	Cell	EC_{50} (μM)	CC_{50} (μM)	SI
BCDS Copper(I)	RSV	MA-104	<5	>250	>50
MP-BCDS Copper(I)	RSV	MA-104	<2	>250	>125
MM-BCDS Copper(I)	RSV	MA-104	<2	>250	>125
BCDS Copper(I)	HBV	2.2.15	2.1	666	28.5
BCDS Copper(I)	IF-A (H1N1)	MDCK	55	>250	>4.5
MP-BCDS Copper(I)	IF-A (H1N1)	MDCK	33	>250	>7.6
MM-BCDS Copper(I)	IF-A (H1N1)	MDCK	34	<145	<4.3

BCDS Copper(I)	IF-A (H3N2)	MDCK	55	>250	>4.5
MP-BCDS Copper(I)	IF-A (H3N2)	MDCK	55	>250	>4.5
MM-BCDS Copper(I)	IF-A (H3N2)	MDCK	45	<149	<3.3
BCDS-Copper(I)	IF-B	MDCK	38	52	1.4
MP-BCDS-Copper(I)	IF-B	MDCK	25	76	3.0
MM-BCDS-Copper(I)	IF-B	MDCK	41	74	1.8

In the HBV assay, HBV virions released by the cells, 2.2.15 (Korba and Milman, Antiviral Res. 15:217,1991), are quantified via DNA hybridization to specific radiolabeled HBV DNA fragments (Korba and Milman, Antiviral Res. 19:55, 1992). In this technique, EC_{50} and EC_{90} are determined as the effective concentration of the test compound which reduces the yield of HBV DNA by 50% and 90%, respectively. The CC_{50} is determined for the test compound on uninfected cells, and the SI is the ratio CC_{50}/EC_{90}.

For the RSV assay, MA-104 cells are cultured in monolayer, and the cytopathic effect of the virus is quantified by vital dye (Neutral Red Uptake). This quantitative method is also used for determination of the CC_{50}. The point of 50% and 90% dye uptake are determined for both the infected cells (EC_{50} and EC_{90}, respectively) and uninfected cells (CC_{50}), and the ratio of CC_{50}/EC_{50} is the SI.

In the IF-A and IF-B assays, Madin-Darby Canine Kidney (MDCK) cells were used in combination with the Neutral Red Uptake technique as described above with regard to the RSV assay.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not to be limited except as by the appended claims.

Claims

1. Use of a stable Copper(I) complex as an active therapeutic substance.

2. A composition comprising a stable Copper(I) complex in combination with a pharmaceutically acceptable carrier or diluent.

3. The composition of claim 2 wherein the stable Copper(I) complex is (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt) Copper(I) (2:1).

4. The composition of claim 3, wherein the stable Copper(I) complex is a single isomer of (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt) Copper(I) (2:1).

5. The composition of claim 2 wherein the stable Copper(I) complex is (2,9-dimethyl-1,10-phenanthroline) Copper(I) (2:1).

6. Use of a stable Copper(I) complex in the manufacture of a medicament for inhibiting viral replication in a warm-blooded animal.

7. The use of claim 6, wherein the virus is selected from the group consisting of human T-cell leukemia I and/or II, human herpes virus, cytomegalo virus, encephalomyocarditis virus, Epstein Barr virus, human hepatitis virus, Varicella Zoster virus, Rhinovirus, rubella virus, respiratory Syncytium virus, influenza virus, parainfluenza virus and adenovirus.

8. The use of claim 6, wherein the virus is human immunodeficiency virus.

9. Use of a stable Copper(I) complex in the manufacture of a medicament for inhibiting infection by a virus in a warm-blooded animal.

10. The use of claim 9 wherein the virus is human immumodeficiency virus.

11. The use of claim 9 wherein the medicament is formulated for topical application.

12. The use of claim 11 wherein the medicament is formulated for topical administration to the epithelium of the vaginal mucosa, cervix, anus or penis.

13. The use of claim 9 wherein the stable Copper(I) complex is a bathocuproine disulfonic acid Copper(I) complex.

14. The use of claim 13 wherein the bathocuproine disulfonic acid Copper(I) complex is selected from bathocuproine-para, para-disulfonic acid Copper(I), bathocuproine-meta, para-disulfonic acid Copper(I), bathocuproine-meta, meta-disulfonic acid Copper(I), bathocuproine-ortho, meta-disulfonic acid Copper(I), and mixtures thereof.

15. The use of claim 13 wherein the ratio of bathocuproine disulfonic acid to Copper(I) is 2:1.

16. Use of a stable Copper(I) complex in the manufacture of a medicament for inhibiting the transmission of sexually transmitted diseases in a warm-blooded animal.

17. The use of claim 16 wherein the sexually transmitted disease is selected from human immunodeficiency virus, human herpes virus and hepatitis virus.

18. The use of claim 17 wherein the medicament is formulated for topical administration.

19. The use of claim 18 wherein the medicament is forumulated for topical administration to the epithelium of the vaginal mucosa, cervix, anus or penis.

20. The use of claim 16 wherein the stable Copper(I) complex is a bathocuproine disulfonic acid Copper(I) complex.

21. The use of claim 20 wherein the bathocuproine disulfonic and Copper(I) complex is selected from bathocuproine-para, para-disulfonic acid Copper(I), bathocuproine-meta, para-disulfonic acid Copper(I), bathocuproine-meta, meta-disulfonic acid Copper(I), bathocuproine-ortho, meta-disulfonic acid Copper(I), and mixtures thereof.

22. The use of claim 20 wherein the ratio of bathocuproine disulfonic acid to Copper(I) is 2:1.

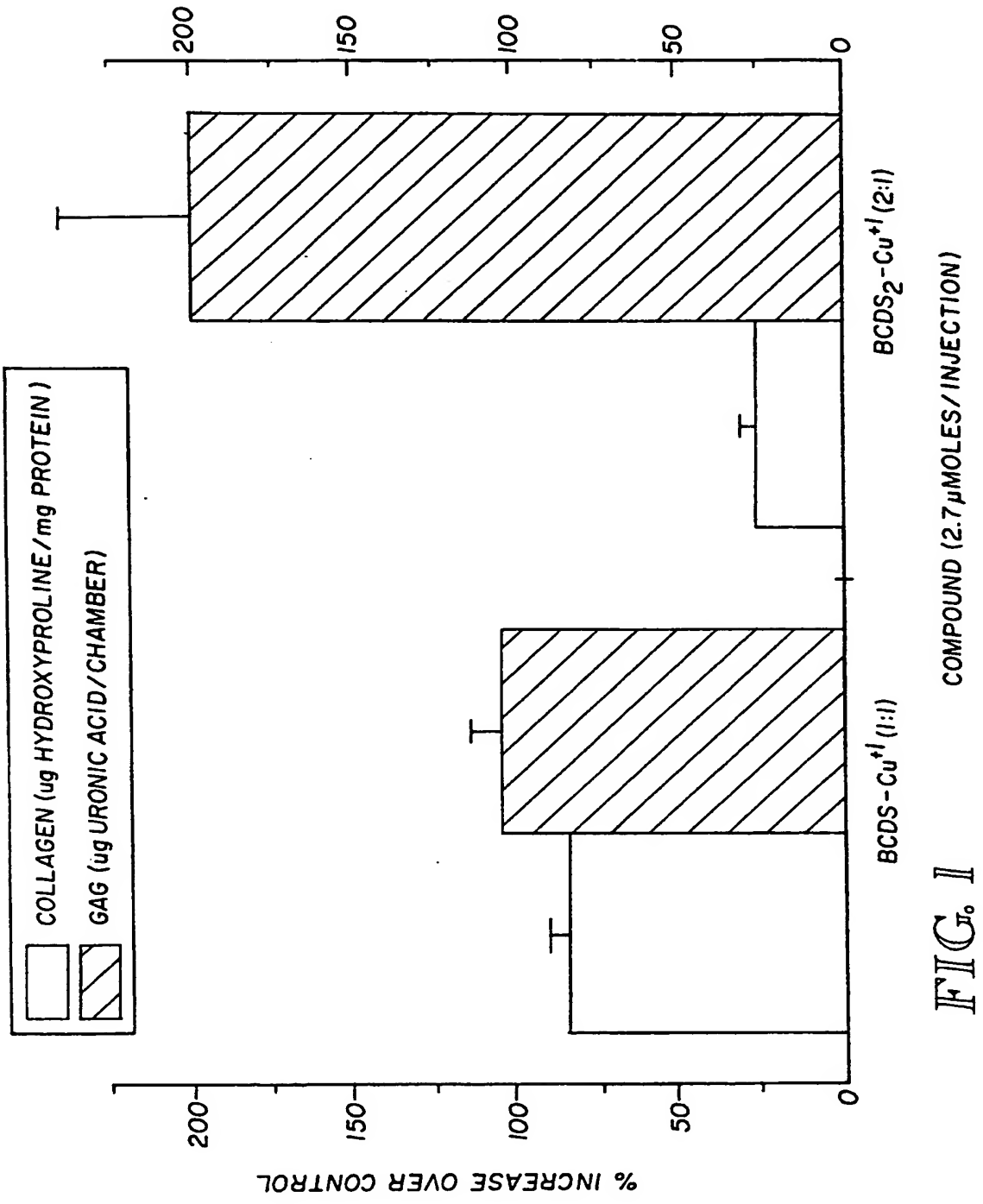
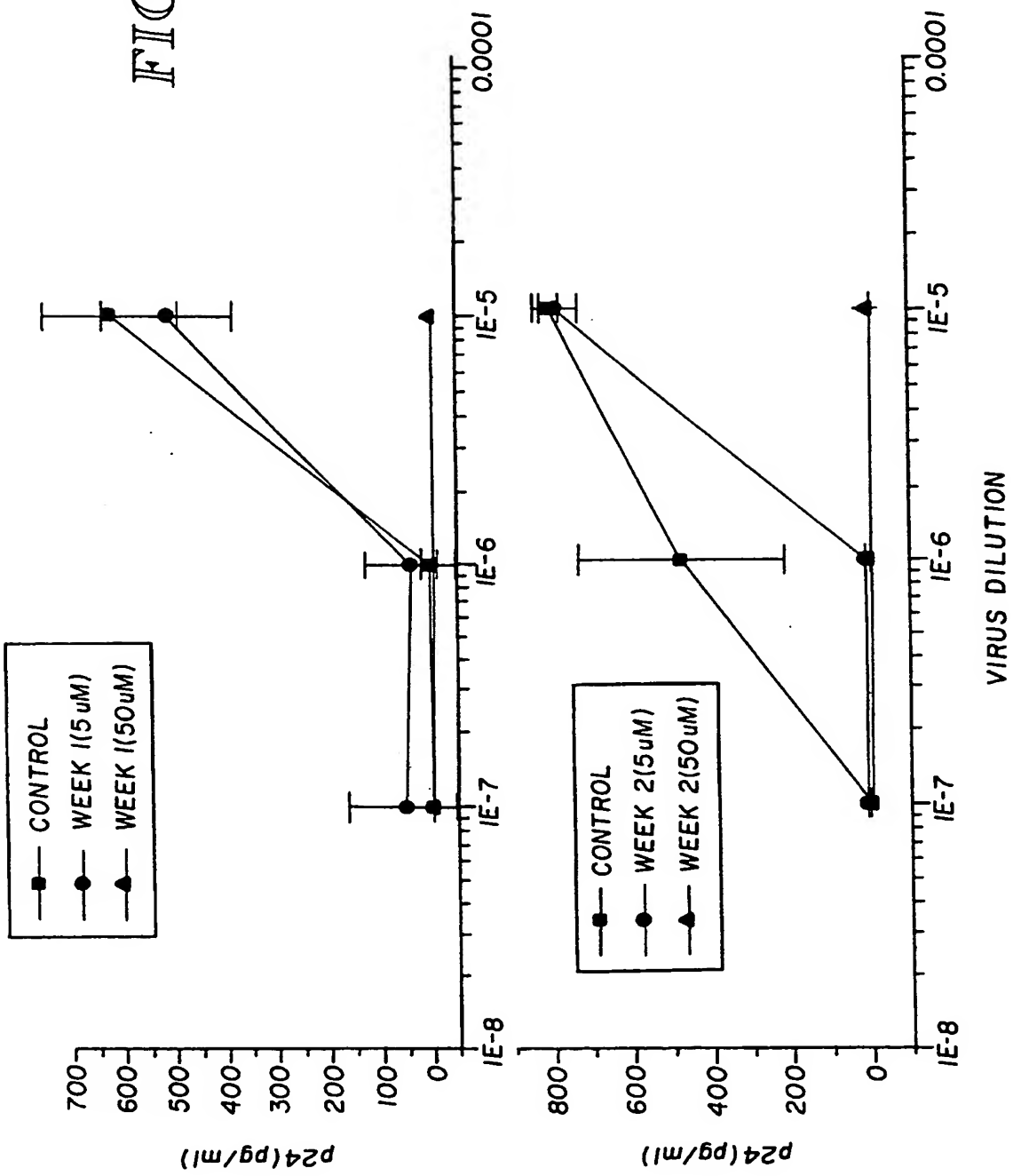

COLLAGEN (ug HYDROXYPROLINE/mg PROTEIN)
GAG (ug URONIC ACID/CHAMBER)
% INCREASE OVER CONTROL
0
50
100
150
200
BCDS-Cu+1 (1:1)
BCDS2-Cu+1 (2:1)
COMPOUND (2.7 μMOLES/INJECTION)

FIG. II

FIG. 2


CONTROL
WEEK 1(5uM)
WEEK 1(50uM)
CONTROL
WEEK 2(5uM)
WEEK 2(50uM)
p24(pg/ml)
p24(pg/ml)
VIRUS DILUTION
0.0001
1E-5
1E-6
1E-7
1E-8
700
600
500
400
300
200
100
0
800
600
400
200
0

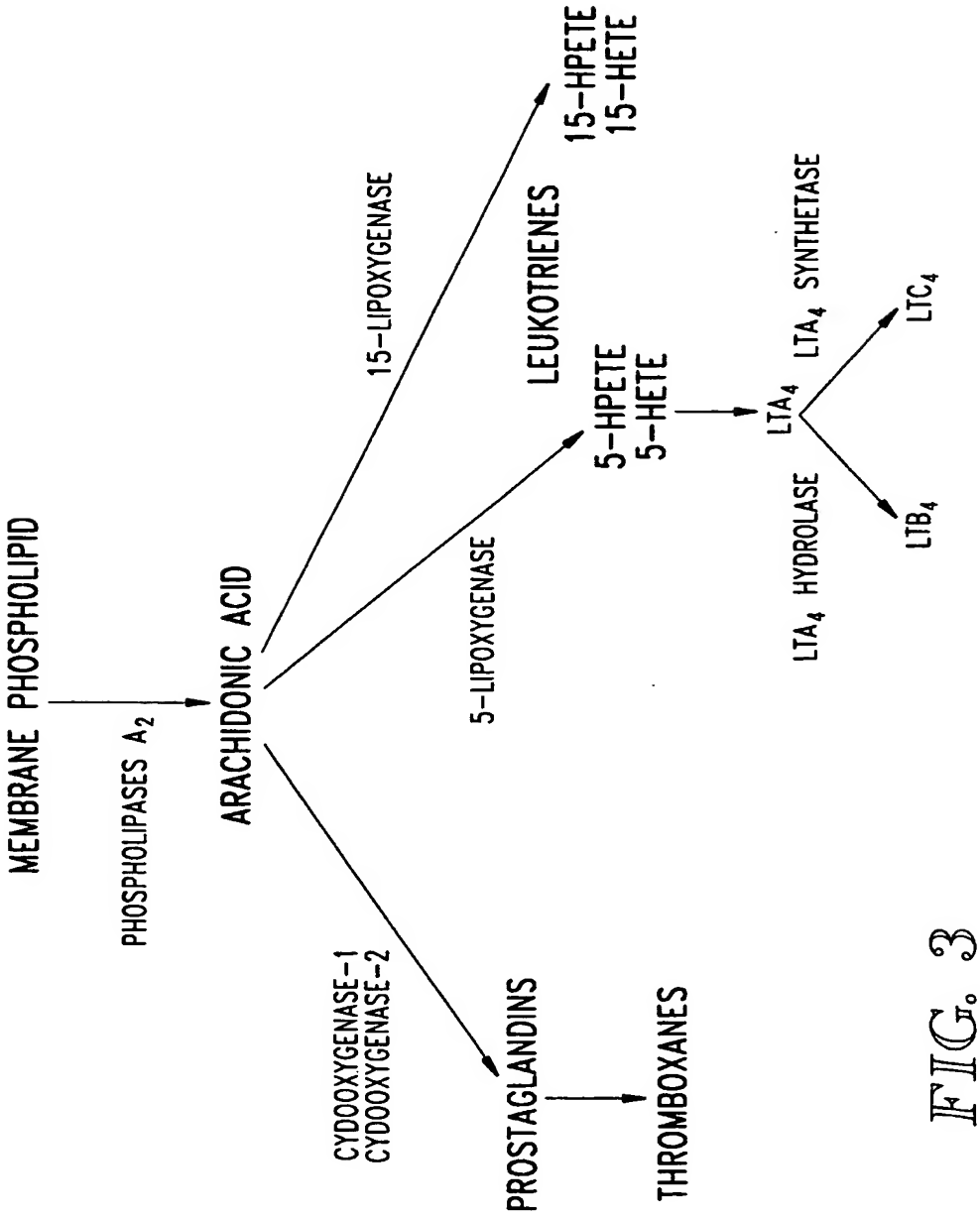

MEMBRANE PHOSPHOLIPID
PHOSPHOLIPASES A_2
ARACHIDONIC ACID
15-LIPOXYGENASE
15-HPETE
15-HETE
LEUKOTRIENES
5-LIPOXYGENASE
5-HPETE
5-HETE
LTA_4
LTA_4 SYNTHETASE
LTC_4
LTA_4 HYDROLASE
LTB_4
CYDOOXYGENASE-1
CYDOOXYGENASE-2
PROSTAGLANDINS
THROMBOXANES

FIG. 3

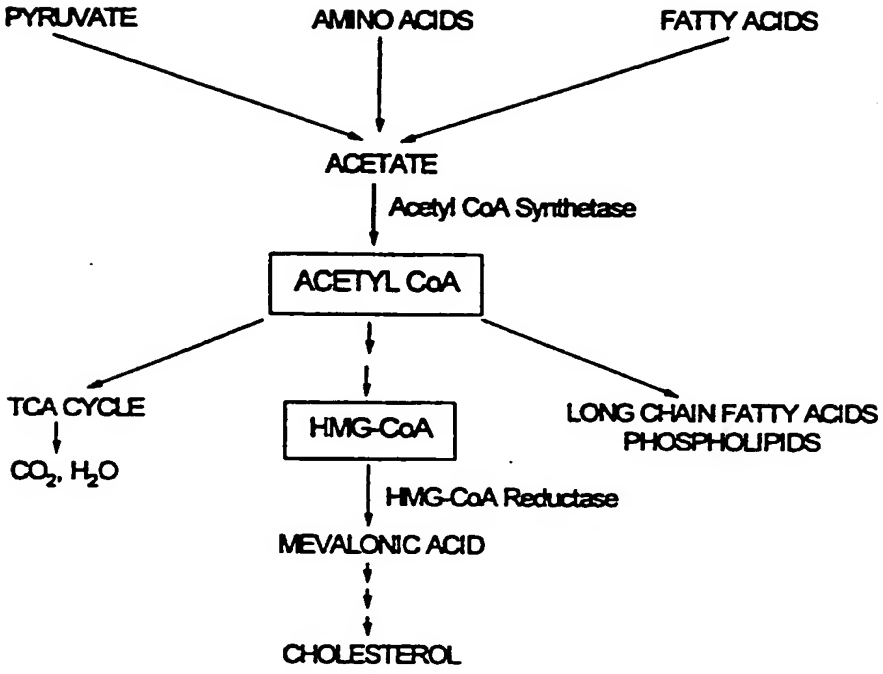

PYRUVATE
AMINO ACIDS
FATTY ACIDS
ACETATE
Acetyl CoA Synthetase
ACETYL CoA
TCA CYCLE
CO_2, H_2O
HMG-CoA
LONG CHAIN FATTY ACIDS
PHOSPHOLIPIDS
HMG-CoA Reductase
MEVALONIC ACID
CHOLESTEROL

FIG. 4

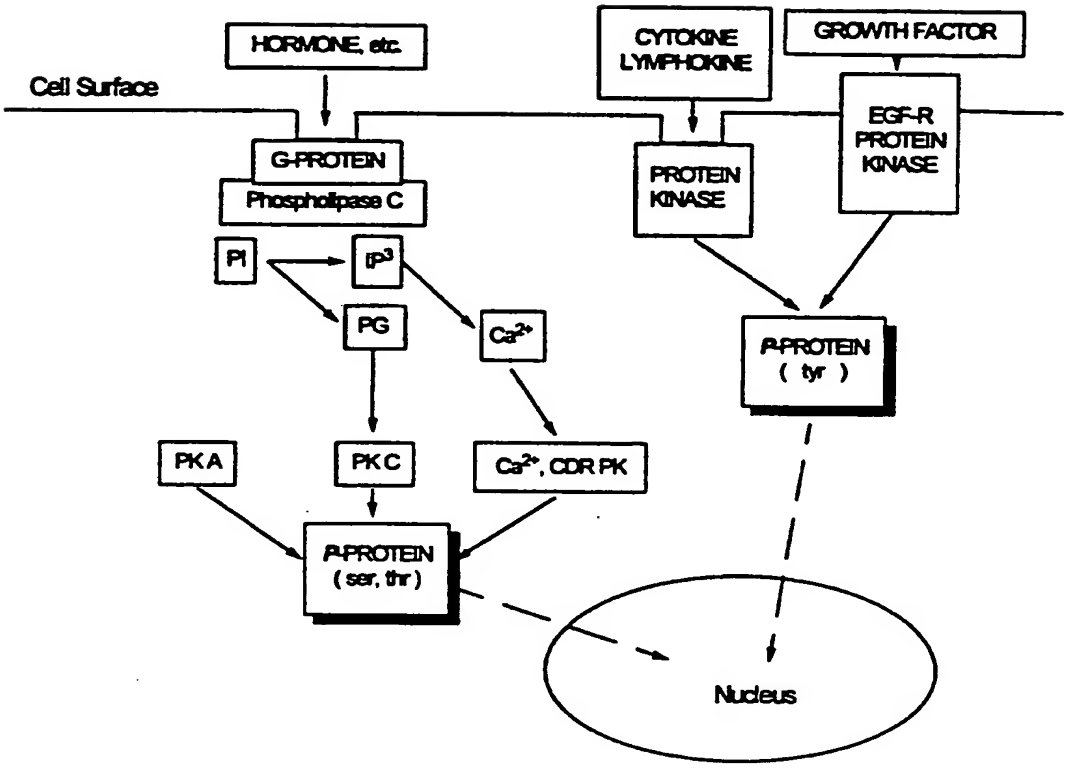

HORMONE, etc.
CYTOKINE LYMPHOKINE
GROWTH FACTOR
Cell Surface
G-PROTEIN
Phospholipase C
PROTEIN KINASE
EGF-R PROTEIN KINASE
PI
IP^3
PG
Ca^{2+}
P-PROTEIN (tyr)
PKA
PKC
Ca^{2+}, CDR PK
P-PROTEIN (ser, thr)
Nucleus

FIG. 5

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/10122

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/47 A61K31/30 A61K31/555

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 322, no. 1, 10 September 1995, pages 127-134, XP002016034 D.A. DAVIS ET AL.: "INHIBITION OF THE HUMAN IMMUNODEFICIENCY VIRUS-1 PROTEASE AND HUMAN IMMUNODEFICIENCY VIRUS-1 REPLICATION BY BATHOCUPROINE DISULFONIC ACID CU1+" see the whole document ---	1-22
P,X	PHARMACEUTICAL RESEARCH, vol. 12, no. 9, September 1995, page S380 XP002016035 G. GENDRON ET AL.: "THE PHARMACOKINETICS AND IN VITRO METABOLISM OF PC1250 (BATHOCUPROINE DISULFONIC ACID:CU(I), 2:1) AND ITS CORRESPONDING FREE LIGAND" see the whole document ---	1-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 October 1996	29.10.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hoff, P

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/US 96/10122

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,94 27594 (PROCYTE CORPORATION) 8 December 1994 see abstract; claims 1-5,14-16; examples 14-17; tables 19-27	1-22
X	AIDS RESEARCH AND HUMAN RETROVIRUSES, vol. 11, no. 1, January 1995, pages 115-125, XP000604986 A. MAZUMDER ET AL.: "INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INTEGRASE BY A HYDROPHOBIC CATION: THE PHENANTHROLINE-CUPROUS COMPLEX" see the whole document, in particular page 124, 4th paragraph	1-22
X	US,A,4 004 006 (SHULMAN ET AL.) 18 January 1977 see the whole document, in particular claim 26	1,2,16
A	WO,A,92 15329 (THE UNITED STATE OF AMERICA) 17 September 1992 see the whole document	1-22
A	CHEM.-BIOL. INTERACTIONS, vol. 6, 1973, pages 407-413, XP002016036 A. SHULMAN ET AL.: "VIROSTATIC ACTIVITY OF 1,10-PHENANTHROLINE TRANSITION METAL CHELATES: A STRUCTURE-ACTIVITY ANALYSIS" see the whole document	1-22

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

In' tional Application No
PCT/US 96/10122

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9427594	08-12-94	AU-A-	7051794	20-12-94
		CA-A-	2163640	08-12-94
		EP-A-	0701439	20-03-96
		ZA-A-	9403857	01-02-95
US-A-4004006	18-01-77	AU-B-	498201	22-02-79
		AU-A-	7525174	13-05-76
		CA-A-	1028949	04-04-78
		DE-A-	2453624	15-05-75
		FR-A-	2250529	06-06-75
		JP-A-	50116615	12-09-75
		SE-B-	431151	23-01-84
		SE-A-	7413904	13-05-75
WO-A-9215329	17-09-92	AU-A-	1463992	06-10-92